LANGERHANS DENDRITIC CELLS, TOBACCO SMOKE AND LUNG TUMOURS

by

NAIEM A. ZEID, M.B.B.Ch.
DEPARTMENT OF PATHOLOGY
UNIVERSITY OF TASMANIA
AUSTRALIA

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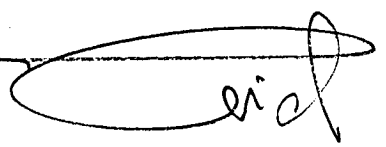
SIGNED: 

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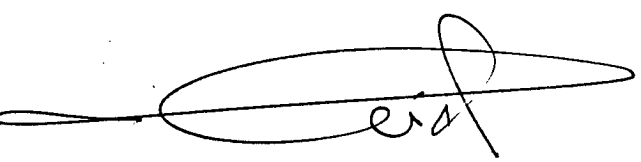

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ABSTRACT

The distribution of Langerhans dendritic cells in tobacco smoke related human lung cancer, tobacco smoke induced Langerhans cells changes during cutaneous and pulmonary carcinogenesis in mice were investigated. While dendritic cells were rare in normal lung, their density was marked in bronchioalveolar, well and moderately differentiated squamous cell carcinomas. Small cell lung cancer and poorly differentiated squamous cell carcinoma showed the lowest density. High density of dendritic cells in lung tumours was associated with marked tumour infiltrating lymphocytes and favourable survival.

Exposure of mice to tobacco smoke increased the density of zinc-iodide-osmium positive pulmonary Langerhans cells. Pulmonary Langerhans cell granulomatosis and a proliferative alveolar and bronchial epithelial tumour-like lesions were observed. The density of pulmonary Langerhans cells returned to that of the control level after ceasing exposure to tobacco smoke and the interstitial granulomatous lesions disappeared, but the bronchial epithelial metaplasia did not reverse. The ultrastructure of the zinc-iodide-osmium positive cells was similar to that of pulmonary Langerhans cells.

Tobacco smoke condensate increased the density, changed the morphology and impaired the function of epidermal Langerhans cells. This was associated with skin tumour development.

While examining human lung lesions S100 positive DC were observed migrating into tumours. So these cells and their relation to LDC were further examined in blood. Metrizamide enriched dendritic cells isolated from human blood, were S100 positive, co-expressed CD45 and were non-phagocytic. Their diameter was larger than that of CD3 positive T lymphocytes and smaller than CD14 positive monocytes. The ultrastructural features were consistent with that of blood dendritic cells. Their number slightly decreased with age, equal numbers were observed in both sexes and formed 0.09 ± 0.07 cells x 10^9 per litre of blood.

PREFACE

The family of Langerhans dendritic cells (LDC) includes epidermal LDC, indeterminate cells in the upper dermis of the skin, veiled cells in the afferent lymph, interdigitating DC (IDC) in lymph nodes and spleen, interstitial DC in various organs and their precursors in the blood.

Antigen presentation, migratory function and T cell activation are the three major functions of LDC. As antigen presenting cells, LDC strongly express all the class-II MHC molecules, (both Ia in mouse and HLA-DP, DQ, DR in human). They can capture antigen from the epithelial surface and present it to T-lymphocytes in the regional lymph nodes.

The anti-cancer activity of bone marrow derived S100 positive LDC has been investigated for adenocarcinoma of the lung, nasopharyngeal carcinomas, gastric carcinomas, papillary thyroid tumours, colorectal carcinomas, oesophageal squamous cell carcinomas, laryngeal carcinoma, prostatic carcinoma, cervical squamous cell carcinoma and transitional cell carcinoma of the urinary bladder. All reports demonstrated an increased survival for patients associated with high density of LDC in these tumours. Their role in tobacco smoke related lung cancer had not been investigated conclusively prior to this study.

Tobacco smoke remains a major cause of lung tumours and chronic pulmonary diseases not only in Australia, but world wide. The annual costs of medical care for smoking related illness for the US and Canada population was more than US$ 501 billion. In West Australia, 13,944 deaths were a result of tobacco smoking between 1981-1990.

The role of LDC in tobacco smoke induced pulmonary lesions is not clearly understood. Though the effect of tobacco smoke derived carcinogens, benzo(a)pyrene and catechols on LDC have been investigated previously during cutaneous carcinogenesis, crude tobacco smoke was not used before to investigate its effect on pulmonary antigen presenting LDC and to examine the relationship between the tobacco smoke induced LDC changes and tobacco smoke induced pulmonary lesions. The role of pulmonary LDC in the development of tobacco smoke induced pulmonary lesions is not clearly understood.

This thesis was designed to examine the role of Langerhans dendritic cells in tobacco smoke related human lung tumours. Antigen presenting $S100^+$ Langerhans dendritic cells were examined in normal trachiobronchial epithelium, lung, normal bronchial lymph nodes, lung tumours and in lymph nodes regional to these tumours. Their density, morphology, contact with tumour cells and TIL and relationship to patient survival was investigated in all varieties of lung cancer.

Crude tobacco smoke was used for the first time in this study to investigate its effect on murine pulmonary antigen presenting LDC and to examine the relationship between the tobacco smoke induced LDC changes and tobacco smoke induced pulmonary lesions. The density of zinc-iodide-osmium positive pulmonary Langerhans dendritic cells was assessed in mice after passive exposure to tobacco smoke. After ceasing exposure to tobacco smoke the density of pulmonary Langerhans cells and their relationship to the pathological changes observed at that stage was studied.

The relationship between the tobacco smoke condensate induced LDC changes and TSC induced skin lesions was also examined.

Characterisation of $S100^+$ cells in human blood in this study arose from observing $S100^+$ cells in blood vessels while examining the $S100^+$ LDC in human lung tumours (Zeid and Muller, 1993). Although these $S100^+$ cells were small, they were observed to increase in size and in the number of dendrites extending from their cell body during their migration through blood vessel wall. It was proposed that these $S100^+$ cells found in the blood vessels were the precursors of the $S100^+$ LDC investigated in lung tumours.

Metrizamide enriched dendritic cells (DC) were isolated from human blood and examined for their cytoplasmic S100 protein, and surface markers. These cells were assesed by image analysis, eyepiece micrometer and their ultrastructure examined and compared with that of T lymphocytes and monocytes.

In summary the aims of this thesis were:

1. To investigate LDC in tobacco smoke related human lung cancer.
2. To study the effect of passive tobacco smoke inhalation on pulmonary LDC in mice.
3. To examine the effect of crude tobacco smoke condensate on murine epidermal LDC during cutaneous carcinogenesis.
4. To study S100 positive cells in the peripheral blood.

FIGURES

TABLES

ABBREVIATIONS

Å	angstrom unit
APC	antigen presenting cell(s)
ATPase	adenosine triphosphatase
BA	bronchioalveolar carcinoma
BG	Birbeck granule(s)
BM	bone marrow
CIN	cervical intra epithelial neoplasia
CTL	cytotoxic T lymphocytes
DAB	diaminobenzidine
DC	dendritic cell(s)
DIP	desquamative interstitial pneumonia
EDTA	tetra-sodium ethylenediamine tetraacetic acid
FDC	Follicular dendritic cell(s)
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G	gram
GM-CSF	granulocyte macrophages-colony stimulating factor
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
HLA-DR	human class II major histocompatibility complex antigen, region DR
HLA-QR	human class II major histocompatibility complex antigen, region QR
HPF	high power field
HX	histiocytosis X
Ia	murine class II major histocompatibility complex antigen
IDC	interdigitating dendritic cell(s)

IFN	interferon
IL-1	Interleukin-1
IL-2	Interleukin-2
LC	Langerhans cell(s)
LDC	Langerhans dendritic cells
LN	lymph node(s)
MHC	Major histocompatibility complex
NKC	natural killer cell(s)
PAS	periodic-acid schiff
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PGE2	prostaglandins E2
PHX	pulmonary histiocytosis X
RNA	ribonucleic acid
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
TAA	tumour associated antigen
TGF	transforming growth factor
TIL	tumour infiltrating lymphocytes
TNCB	2,4,6-trinitrochorobenzene
TNF	tumour necrosis factor
TS	tobacco smoke
TSC	tobacco smoke condensate
WBC	white blood cell(s)
ZIO	zinc-iodid-osmium
µm	micron
µl	microlitre

CHAPTER 1

LITERATURE REVIEW

LANGERHANS DENDRITIC CELLS, RESPIRATORY SYSTEM AND TOBACCO CARCINOGENESIS

1. Langerhans Dendritic Cell

1.1 Introduction

Paul Langerhans was a second year medical student in Berlin, Germany in 1868 when he published his discovery of non-pigmentary dendritic cells in the skin. He discovered this cell using a new gold chloride stain investigated by his teacher Cohnheim to demonstrate the peripheral nerve endings in the epidermis of human skin (Ebling, 1980). Langerhans considered these dendritic cells in the epidermis to be peripheral nerve endings because they stained with gold chloride.

Masson, (1947) proposed that the Dopa negative high level clear epidermal cells, described by Langerhans, were aged melanocytes in the process of exfoliation. Billingham, (1948) supported the theory of Masson. Ferreira-Marques, (1951) considered Langerhans dendritic cells (LDC) as part of cutaneous innervation. The Langerhans dendritic cell/melanocyte hypothesis remained a controversial issue until 1961, when Birbeck and co-workers reported the discovery of a unique organelle, the Birbeck granule (BG) in the cytoplasm of LDC which was not observed in melanocytes or keratinocytes (Birbeck et al., 1961).

1.2 Origin of Langerhans dendritic cell

The bone marrow was first demonstrated in 1979 as the site of origin of epidermal LDC by Frelinger et al., (1979) and Katz et al., (1979). Using bone marrow transplantation following X-ray irradiation, the Ia positive LDC in the recipient skin were found to be derived from the bone marrow of donor mice.

Tamaki et al., (1980) also transplanted bone marrow to chimaeric mice confirming the bone marrow origin of LDC, while Pelletier et al., (1984) and Volc-Platzer et al., (1984) were the first to demonstrate the bone marrow origin of LDC in humans. After bone marrow transplantation from a donor male to a female recipient they found that LDC in the skin of a female recipient contained a Y chromosome indicating that these cells could only have been derived from the transplanted marrow.

While bone marrow has been confirmed as the origin of LDC, the progenitor cell has yet to be identified. Clark and Kamen, (1987) proposed three possibilities for the progenitor of LDC. Firstly, the stem cell which generates the lymphoid lineage, secondly the stem cell which differentiates into monocytes and thirdly the stem cell of LDC may be a completely separate lineage.

Because of a similarity in surface markers many reports have suggested the monocyte as the progenitor of LDC. LDC and monocytes have receptors for C3 and the Fc portion of IgG, and both have class II major histocompatibility antigens (Rowden et al., 1977; Stingl et al., 1977;

Barfoot et al., 1989). Against the monocytic origin of LDC, the latter are S100, CD1a and HLA-DQ positive while monocytes are S100, CD1a and HLA-DQ negative (Furukawa et al., 1984). Furthermore, LDC lack non-specific esterase, lysozyme and the YI/82A marker of monocytes-macrophages (Reid et al., 1990). Whether monocytes and LDC arise from a common stem cell in the bone marrow awaits clarification (Jaffe, 1993).

1.3 Identification of Langerhans dendritic cells

Gold chloride was first used to stain LDC in the skin (Langerhans, 1868; Breathnach, 1965). While LDC can not be observed in routine paraffin embedded, haematoxylin and eosin (H&E) stained sections, they can be identified by means of enzyme histochemistry, cell surface antigens, cytoplasmic reactions including S100 and zinc-iodide-osmium (ZIO) impregnation and conclusively by electron microscopy examination to demonstrate BG (Birbeck et al., 1961).

1.3.1 Enzyme histochemistry

The cytoplasm of LDC contains a variety of hydrolytic enzymes which can be utilised by enzyme-histochemical techniques to detect LDC (Wolff, 1972). These hydrolytic enzymes include alkaline phosphatase (Kechijian, 1965), nucleosidephosphatase, such as adenosine triphosphatase (ATPase), which is found in the epidermal LDC of human, guinea pigs, mice and monkeys (Wolff, 1972), aminopeptidase, in LDC of guinea pigs (Wolff, 1964) and cholinesterase, found in the high level dendritic cells of the sheep epidermis (Lyne and Chase, 1966). Non-

specific esterase, are observed in LDC of rats and mice (Wolff, 1972), acid phosphatase in the Golgi apparatus and lysosomes of LDC, and endogenous peroxidase, in LDC of murine epidermis (Maruyama et al., 1980). ß-Glucuronidase is an excellent marker for LDC from mouse, human, monkey, guinea pig, rabbit and rat epidermis (Mackenzie et al., 1982).

1.3.2 Cell surface antigen markers

LDC share some of the lymphoid cell surface markers, such as receptors for C3, Fc IgG (Stingl et al., 1977), OKT6 (CD1) and HLA-DR antigen (Ia in mouse and rat, Rowden et al., 1977). Various monoclonal antibodies react specifically with these surface markers which help to identify LDC in tissues. The best known surface markers of LDC commonly used to identify this cell are, HLA-DR antigen in human LDC, Ia antigen in mice (Rowden et al., 1977), OKT-6 (CD1) antigen in humans (Murphy et al., 1981), receptors for C3 and the Fc portion of IgG in humans (Stingl et al., 1977).

Steinman, (1991) reported that LDC expressed the following surface markers: MHC class I and MHC class II, leucocyte common antigen (CD45), B cell marker (CD40), T cell markers (CD1, CD4, CD8), ß2 Integrins (CD11a, CD11b and CD11c), adhesion molecules (CD54, CD58), and other receptors such as receptor for IL-2 (CD25). LDC were also reported negative for NK cell markers (CD56, CD57).

Other surface markers also have been reported e.g. B7-1, B7-2 integrins CD80, CD86 (Inaba et al., 1994) and CD68 (Van Haarst et al., 1994). The expression of LDC for the leucocyte common antigen, some surface markers of both T and B cell may suggest that LDC originate from a multilineage rather than a single-lineage bone marrow stem cell (Furuta et al., 1993).

1.3.3 Cytoplasmic markers

The cytoplasm of human LDC contains S100 protein (Cocchia et al., 1981). This protein, called S100 because of its solubility in 100% ammonium sulphate, was discovered in 1965 in cells of the human nervous system (Moore, 1965). It was later found in other cell types including, chondrocytes, myoepithelial cells, melanoma cells and interdigitating dendritic cells in lymph nodes (Hammar et al., 1986). The S100 protein is an acidic, calcium-binding protein with molecular weight between 21,000 and 24,000 Dalton. It consists of a single tryptophan, three tyrosine, three or four cysteines and a number of phenylalanine residues (Moore, 1965). Though the biological role of S100 protein is not fully known, it has been reported that S100 protein consisted of two subunits α and β (Isobe et al., 1983). S100α protein and not S100β can inhibit the enzymatic activity of glycogen phosphorylase a in a calcium-independent manner (Zimmer and Dubuisson, 1993).

LDC do not contain the lysozymes (Furukawa et al., 1984) which are classically seen in the cytoplasm of monocytes-macrophages.

The zinc-iodide-osmium (ZIO) procedure was originally reported by Champy, (1913) to visualise chromaffin granules of nerve cell synaptic vesicles. While Niebauer et al., (1969) utilised the ZIO procedure to detect epidermal LDC and reported the affinity of BG for the ZIO stain, Wolff, (1972) reported both LDC and histiocytosis X cells had the affinity for the ZIO stain. Rodriguez and Caorsi (1978) used the ZIO procedure to visualise LDC and its characteristic structural marker, the Birbeck granule. The internal structure of BG is selectively stained with this procedure. This has been used to proceed from light to electron microscopy examination of LDC in various tissues eg. to study LDC in human epidermis (Rodriguez and Caorsi, 1978) and uterine cervix (Caorsi and Figueroa, 1986).

1.4 Ultrastructure of Langerhans dendritic cells

The first electron microscopy description of the ultrastructure of LDC in human epidermis was in 1958 by Clark and Hibbs, (1958). Birbeck and his colleagues (1961), subsequently reported a unique cytoplasmic organelle in LDC but not other epidermal cells. Since then this cytoplasmic organelle, has been considered as the ultimate structural marker of LDC. Its major ultrastructural features are the rod and the disc forming the characteristic tennis racket shape organelle (Zelickson, 1965), with its trilaminar limiting membrane (Birbeck et al., 1961).

1.4.1 Morphology and size of Langerhans dendritic cell

The morphology of the cell body of LDC has been described as triangular, oval or rounded in shape, 8-20 μm in diameter. Various numbers of dendrites extend from the plasma membrane giving the cell its characteristic dendritic nature (Rodriguez and Caorsi, 1978; Chu et al., 1983).

There are no desmosomal attachments between the plasma membrane of LDC and the adjacent epidermal cells (Birbeck et al., 1961; Younes et al. 1968 and Wolff, 1972). This may reflect the migratory nature of these cells.

The number, length and the branching nature of the dendrites vary from cell to cell. The length of these dendrites may reach up to 30 nm (Rodriguez and Caorsi, 1978). Figueroa and Caorsi, (1980) subdivided Langerhans cells into five types according to the number of dendrites: type I, one unbranched dendrite; type II, one branched dendrite; type III, two branched or unbranched dendrites; type IV, three dendrites and type V, more than three dendrites and with numerous branches.

The cytoplasm of LDC is clear in the suprabasal layer of the epidermis of skin. This is due to the absence of keratin tonofilaments (Birbeck et al., 1961). The clear cytoplasm of LDC has been confirmed by many investigators (Zelickson, 1965; Younes et al., 1968; Wolff, 1972; Hammar, 1988).

The nucleus of LDC is large, indented, lobulated or convoluted with a prominent nucleolus (Hammar, 1988). LDC with rounded nuclei have been reported by Wolff, (1972). The nucleus is darker than that of the surrounding epidermal cells (Younes et al., 1968). Zelickson, (1965) reported the presence of holes in the nuclear membrane after electron microscopy examination and thought they represented a direct communication between the nucleus and the cytoplasm.

1.4.2 Cytoplasmic organelles

The cytoplasm contains many organelles found in biologically active cells. These include, mitochondria, Golgi apparatus, smooth and rough endoplasmic reticulum, centrioles and ribosomes. The unique ultrastructural marker, the Birbeck granule (Birbeck et al., 1961; Wolff, 1972; Hammar, 1988) is also commonly identified in the cytoplasm.

The mitochondria of the LDC are similar to those of most cells. The number of mitochondria in the cytoplasm has been reported as not numerous by Birbeck et al., (1961), while Wolff, (1972) and Hammar, (1988) reported the mitochondria as moderate in number.

The LDC Golgi apparatus is well developed, frequently more than one and located on one side of the nucleus. It consists of many cysternae and multiple small vesicles. BG are commonly found in the region of Golgi complex. Some workers suggested that BG are secreted by the Golgi apparatus (Breathnach, 1964).

The cytoplasm of LDC contains centrioles in the region of Golgi complex and around the nucleus (Birbeck et al, 1961; Wolff, 1972). Thus LDC have the structural elements needed for cell division. Mitotic division of LDC remains a controversial hypothesis. Giacometti, (1969) studied skin during wound healing and reported LDC labelled with tritiated thymidine were found 2, 5, 6, 7, and 16 days after wounding. Giacometti, (1969) thought LDC may undergo cell division during the process of wound healing. Czernielewski and Demarchez, (1987) used anti-BrdU (bromodeoxyuridine) and found 6% of epidermal LDC in S-Phase. This suggests that the majority of epidermal LDC are not dividing locally in the skin.

Fine and rough endoplasmic reticulum are organised in a parallel fashion in the cytoplasm of LDC (Hammar, 1988). Ribosomes are involved in the synthesis of the RNA and other cellular protein. Their number in the cytoplasm affect the LDC density in relation to the surrounding keratinocytes. Dark LDC with numerous ribosomes were reported by Ebner and Niebauer, (1967).

In general the cytoplasm of LDC does not contain the large lysosomes or phagocytic vacuoles commonly found in macrophages (Hammar, 1988). Zelickson, (1965) described the ultrastructure of LDC lysosomes as dense, rounded bodies surrounded by a single membrane on the outside, but contain a series of concentric membranes. According to Zelickson, the lysosomes and the lipid droplets are more numerous in the superficially located LDC in the epidermis. He also observed that the plasma membrane of the superficially located LDC became more convoluted

giving the cell the pseudovacuolated appearance (Zelickson, 1965). The increase in the number of lysosomal and lipid droplets together with the changes in cell membrane of the superficially located LDC may represent exfoliation steps of these cells as they pass through the epidermis.

1.4.3 Birbeck granules (BG)

BG are the ultimate ultrastructural marker of LDC (Birbeck et al., 1961). The apparent size and shape of BG depends on the angle at which the LDC have been cut by the microtome. Reported as rod and disc in shape by Birbeck et al., (1961), Zelickson, (1965), described these granules as tennis racket in shape. The granules are about 40 nm in diameter (Hashimoto and Tarnowski, 1968).

BG are composed of trilaminar membranes, the limiting membrane being about 60 Å thick and lined from the inside by small particles arranged at regular spaces of 50-60 Å (Wolff, 1967). In the middle between the two limiting membranes is a third dense line showing a regular periodic striation of 50-60 Å (Zelickson, 1965). The centre of the disc portion of the granules is clear and lined with similar particles lining the rods (Wolff, 1967).

BG are commonly found in the region of the Golgi complex (Wolff, 1972), and less frequently related to the plasma membrane (Breathnach, 1964). BG can be labelled specifically by the zinc-iodide-osmium (ZIO) procedure, (Niebauer et al., 1969; Wolf, 1972; Rodriguez and Caorsi, 1978).

The origin of BG and its function are unclear. There are two theories proposed for the origin of BG in LDC. The first is known as the secretion theory and suggests that BG are secreted by the Golgi apparatus and then move to the cell membrane to release its contents to the extracellular space. The secretory theory was suggested by Breathnach, (1964), and supported by Zelickson, (1966); Wolf, (1967) and Niebauer et al., (1969). The latter used the ZIO procedure to fix and stain epidermal LDC and observed that the Golgi complex and the BG both took up the metal deposit suggesting that they were closely related.

The second theory is known as the endocytosis theory. Various workers have reported that BG are formed by an infolding of the cell membrane (Hashimoto and Tarnowski, 1968). Using electron microscopy, to examine serial sections of human skin, Ishii et al., (1984) observed the steps of coated vesicle formation and invagination from the cell membrane of LDC to form BG. The endocytosis theory may implicate the involvement of BG in processing the antigen by LDC. This is supported by Senoo et al., (1993) , Solcia et al., (1993), and Reis e Sousa et al., (1993).

1.5 Langerhans dendritic cells and other dendritic cells

Thy-1 positive dendritic cells, follicular dendritic (FDC) and dendritic macrophages, all are dendritic cells, but are unrelated to LDC.

1.5.1 Thy-1 positive epidermal dendritic cells

Thy-1 positive dendritic cells were reported in the skin for the first time in 1983 by Tschachler et al., (1983), and they are not related to LDC. They express the Ly5 mouse haematopoietic cell surface marker and are derived from bone marrow. Thy-1 positive DC also express the asialo-GMI surface marker and share other cell markers belonging to natural killer cells (Hamilos, 1988). Unlike LDC they are negative for the plasma membrane Ia antigen (Bergstresser et al., 1985). Recently it was reported that 15% of the CD3 positive T cells in the peripheral blood are Thy-1^+ (Hozumi et al, 1994).

1.5.2 Follicular dendritic cells (FDC)

Humphrey, (1981) reported that FDC are Fc and C3 receptor positive but Ia negative. They are located in the follicular germinal centre of the spleen and lymph nodes, interact with B-lymphocytes and not T-lymphocytes. As the FDC lack surface Ia antigen, are related to B and not to T-lymphocytes, Humphrey, (1981) suggested they do not belong to the Ia positive LDC.

The bone marrow origin of the follicular dendritic cells and their relationship to the monocytic/macrophage lineage was suggested by Fliedner et al., (1990). They suspended the monoblastic cell line THP-1, the histiocytic cell line U-937 and peripheral blood mononuclear cells (PBMC) in different dilutions of RPMI 1640 culture media mixed with 10% FCS. The FDC-specific antigen Ki-M4 was induced only in the

monoblastic cell line, THP-1. They concluded that follicular dendritic cells are related to the mononuclear-phagocytic cell line derived from the bone marrow. Recently, the latter was supported by Maclennan, (1994); Butch et al., (1994) and by Imazeki et al., (1994).

1.5.3 Dendritic macrophages

Barfoot and his colleagues (1989) described a population of DC isolated from the peripheral lymph of the skin, liver of sheep and from the intestinal lymphatics of rats. These DC express class II major histocompatibility antigen on their surfaces and can phagocytose latex beads and influenza virus. Barfoot et al., (1989) suggested dendritic macrophages are related to mononuclear-phagocytes. While LDC are S100 positive, dendritic macrophages are negative for this cytoplasmic protein (Zeid and Muller, 1993). As both the dendritic macrophages and the follicular DC (Fliender et al., 1990) are of monocytic lineage, the relationship of these two cells to each other needs to be clarified.

1.6 Family of Langerhans dendritic cells

LDC of the skin was the first known DC (Langerhans 1868). Steinman, (1991) used the term "the dendritic cell system" to describe all cells related to LDC in the skin, this included veiled cells in the afferent lymph, interdigitating DC (IDC) in lymph nodes and spleen, interstitial DC in various organs and their precursors in the blood.

1.6.1 Indeterminate cells

These cells represent the migratory form of LDC, either leaving the epidermis via the afferent lymphatics or a new generation arriving in the epidermis from their precursors in blood (Macatonia et al., 1987). It is noteworthy that Jarrett, (1963) described some ATPase positive DC in the upper dermis of the skin but he did not investigate this cell further. While human epidermal LDC are OKT-6$^+$ (CD1a) and HLA-DR$^+$, Czernielewski et al., (1983) reported that the indeterminate cells in the dermis are OKT-6$^-$ and HLA-DR$^+$. LDC may change surface markers as they migrate.

1.6.2 Veiled dendritic cells

These cells are found in the afferent lymphatics of the skin and are believed to be LDC migrating to the regional lymph node after exposure to antigen (Balfour et al., 1981). Silberberg et al., (1976), were the first to report LDC in a dermal lymphatics of the skin. Drexhage et al., (1979) reported the resemblance of veiled cells isolated from the peripheral lymph of pigs to the epidermal LDC. Balfour et al., (1981) hypothesised that the epidermal LDC become veiled dendritic cells in the afferent lymphatics and in lymph nodes they differentiate into the interdigitating dendritic cells in the T-dependent area (paracortex). Pugh et al., (1983) isolated DC from the peripheral lymph of rats and supported the observation of Drexhage and co-workers, (1979) that these cells closely resemble the lymphoid DC isolated from mice by Steinman and Cohn, (1973).

1.6.3 Interdigitating dendritic cells

Steinman and Cohn, (1973) described a new generation of non-phagocytic DC in the spleen, lymph nodes and in the intestinal Peyer`s patches. The surfaces of these cells closely interdigitate with T lymphocytes and have since been called interdigitating dendritic cells (IDC). IDC were described in the white pulp of the human spleen by Heusermann et al., (1974) and in the thymus by Toro and Olah, (1966) and confirmed by Kaiserling et al., (1974). In an experimental model Kripke et al., (1993), reported that these cells are Ia^+, may contain BG and they are in the lymph nodes to initiate the immune response. In human they are $S100^+$ (Zeid and Muller, 1993) and BG rarely observed in their cytoplasm (Steinman, 1991).

1.6.4 Interstitial dendritic cells

Hart and Fabre (1981) described the Ia^+ dendritic cells found in the interstitial connective tissue of various organs such as heart, liver, lung and in the mouse intestine. These interstitial dendritic cells are similar to LDC. They are bone marrow derived, strongly MHC class II positive, poorly phagocytic, capable of stimulating resting T lymphocytes, involved in solid organ graft rejection and have cytochemical features similar to those of the LDC (Hart and McKenzie, 1990).

1.7 Anatomical distribution of Langerhans dendritic cells in human body

While LDC were discovered in the human skin almost 127 years ago (Langerhans, 1868), their widespread distribution only became apparent during the 1980's (Hart and Fabre, 1981; Nakajima et al., 1982; Watanabe et al., 1983; Takahashi et al., 1984; Hammar et al., 1986).

Chen and his colleagues (1985), studied the distribution of ATPase-positive LDC in 117 specimens of normal human skin and mucosa taken from different areas of the body. They reported the highest density of LDC in the skin of the face and the neck (976 ± 30.93/mm^2). The lowest density was in the skin of the palm and sole (267 ± 56.14/mm^2 and 189 ± 19.15/mm^2 respectively) while in trunk skin the density was 740 ± 28.97/mm^2, scalp (693 ± 69.56/mm^2) and arm or leg skin (640 ± 40.95/mm^2).

The density of epidermal LDC in experimental animals varies (Bergstresser et al., 1980). In guinea pig, hamster and mouse the LDC density ranged between 600 and 1500 cells/mm^2. While the density was the lowest in the mouse tail (110-269/mm^2), it was the higher in the dorsal skin of these animals. Their density in ear skin and footpads was less than that in dorsal skin.

As LDC are associated with the epithelia in the human body, many researchers have investigated LDC in the ocular epithelium. Rodrigues et al., (1981), reported LDC in the normal conjunctiva and in the periphery of the cornea. The centre of the cornea lack LDC (Chen et al.,

1985). LDC have been reported in cases of allergic conjunctivitis (Tagawa et al., 1983; Takeuchi et al., 1985, 1986), where their pathophysiological role needs to be fully investigated.

The density of LDC in the oral mucosa shows regional variation (Chen et al., 1985), where their density ranged from 420-802 cells/mm^2.

There are few reports in the literature on LDC in the gastrointestinal tract. LDC have been identified in normal esophagus (Yassin et al., 1979) and in carcinoma of the esophagus (Morris et al., 1986 and Matsuda et al., 1990). Tsujitani et al., (1987) investigated the density of $S100^+$ LDC in gastric carcinomas, while Ambe et al., (1989) investigated their density in colorectal carcinomas. LDC were reported in the liver by Hart and Fabre, (1981), while Bock, (1983) identified LDC with BG in the epithelium of the main pancreatic duct of a cat.

Younes and his colleagues (1968) were the first to identify the ultrastructure of LDC in normal human cervix and proposed that the number of LDC was higher in the cervical epithelium of patients with intra-epithelial neoplasia. Figueroa and Caorsi, (1980) investigated the morphology, density and ultrastructure of LDC in the normal cervix using the ZIO procedure. They reported an LDC density between 6.29-10.19 cells/mm^2.

Basset and colleagues (1974) described the ultrastructure of cells similar to LDC in the bronchioalveolar lavage fluid from patients with bronchioalveolar carcinoma of the lung. Sertl and colleagues (1986),

Holt and Schon-Hegrad, (1987) reported that Ia positive dendritic cells represent 1% of all epithelial cells of normal mice and human lungs. Richard et al., (1987) reported $S100^+$ dendritic cells with BG in human bronchial epithelium and supported the above reports in regard to their density.

Colver and colleagues, (1988), described $T6^+$ dendritic cells in the human bladder and they questioned a possible relationship to cutaneous sensitization.

1.8 Functions of Langerhans dendritic cell

Three aspects of LDC functions will be discussed: antigen presentation; LDC migratory function and T cell activation.

1.8.1 Antigen presentation

As antigen presenting cells, LDC strongly express all class-II MHC molecules in mouse and HLA-DP, DQ, DR in human (Steinman, 1991). Macatonia et al., (1987) investigated the antigen presenting function of the epidermal LDC, 30 minutes after skin painting with the contact sensitizer fluorescein isothiocyanate (FITC). Using a fluorescent microscope, FITC was detected on the DC in the draining lymph nodes with the peak of fluorescence intensity at about 24 hours. They concluded that LDC captured the antigen from the skin and presented it to T-lymphocytes in the regional lymph nodes. Kripke et al., (1990), sensitized the skin with FITC and examined the $FITC^+$ draining cells in

the regional lymph nodes by immunofluorescence and immunoelectron microscopy. All of the $FITC^+$ cells were reported Ia^+ and some contained BG in their cytoplasm. Kripke and co-workers reported that Ia^+, $FITC^+$ cells in the regional lymph nodes are LDC, leaving the skin to initiate hypersensitivity response. Various cytokines can affect antigen presentation by LDC, Grabbe et al., (1992) reported that granulocyte-macrophage/colony-stimulating factor (GM-CSF), Interleukin-1 alpha (IL-1 alpha), tumour necrosis factor-alpha (TNF alpha), transforming growth factor-ß (TGF beta) and interferon-gamma (IFN gamma) can up modulate the antigen presentation of LDC.

1.8.2 Migratory function

From the site of their origin in the bone marrow (Katz et al., 1979), the precursors of LDC migrate through the blood to various organs (interstitial dendritic cells) in the body (Steinman, 1991) including the skin. The migratory function of epidermal LDC was investigated by Bujdoso et al., (1989). After intradermal injection of antigen, veiled dendritic cells were recovered from the afferent lymphatics and found to be carrying antigen. When assessed these cells were capable of triggering antigen-specific T-lymphocytes. The veiled LDC have a high level of MHC class-II and have been isolated from the afferent lymphatics of lymph nodes in rabbit, pig, rat, mouse, sheep and human (Steinman, 1991). When LDC reach lymph nodes, they are called interdigitating dendritic (IDC) cells and are found in the T-zone around the germinal follicles. It is not clear what happens to IDC after stimulating the resting T-lymphocytes in the lymph nodes. While there is no evidence for LDC

degeneration in lymph nodes, the cells may exfoliate through the epithelial surfaces in the body (Chen et al., 1985). Cumberbatch and Kimber, (1992), reported TNF-alpha can stimulate the migration of epidermal LDC to the regional lymph nodes to initiate a cutaneous immune response.

1.8.3 T-lymphocyte activation

The process of T-lymphocyte activation by Langerhans dendritic cells is not fully understood. Many workers have investigated this and reported different factors involved in this process. It is known that IL-1 is essential for T-lymphocyte activation, Steinman, (1991) and Sauder, et al., (1984) reported that LDC can produce IL-1. LDC form clusters with T-lymphocytes during primary interaction (Landry et al., 1990), which is essential for the LDC to stimulate the resting T cells (Muller et al., 1994). Landry et al., (1990) reported that the adhesion molecules ICAM-1 and LFA-3 were expressed by the LDC which may be involved in clustering with T-lymphocytes. Steinman, (1991) has proposed that activating T-lymphocytes by LDC may be related to the various cytokines or by membrane binding between T-lymphocytes and LDC. Moulon et al., (1991) investigated the effect of anti-CD1a monoclonal antibody on T cell proliferation and proposed that the CD1a molecule of epidermal LDC may have an important role in T cell activation. While prostaglandins would inhibit T cell proliferation (Kumar and Das, 1994), it was reported by Demidem et al., (1991), that GM-CSF would enhance stimulation of T cell by LDC. Larsen et al., (1994), proposed that GM-CSF upregulated the expression of both B7-1 and B7-2 on LDC and this will stimulate T cells

proliferation. Retinoic acid was also reported upregulate LDC antigen presentation and T cell activation by enhancing the surface expression of HLA-DR, CD11c and ß2 integrin (Meunier et al., 1994).

1.8.4 Activation of Langerhans dendritic cell function and viability

Cytokines like GM-CSF can enhance the function and the viability of LDC (Larsen et al., 1994). Koch et al., (1990) reported cachectin-TNF to enhance the viability but not the function of LDC. In contrast Inaba et al., (1988) reported IL-1 enhances their function, but not the viability of these LDC in vitro culture. While Bagot et al., (1994), reported the immunosuppressive effects of vitamin D on epidermal LDC, Meunier et al., (1994) reported retinoic acid upregulation of LDC antigen presentation.

1.8.5 Role of Langerhans dendritic cells in various human diseases

LDC are involved in the following human diseases:

1. Contact dermatitis, where LDC can take up and present antigen to T cells to generate an immunological response (Shelley and Juhlin, 1976 & Silberberg-Sinakin et al., 1980). If this function of LDC could be modified, this may help to counter this disorder.

2. Hypersensitivity reactions- Fokkens et al., (1990) reported that LDC bear IgE surface molecules in allergic rhinitis and atopic dermatitis. The IgE positive LDC may capture the antigen and present it to T-lymphocytes inducing an allergen-specific T cell response and IgE

synthesis. The role of IgE bearing LDC in allergic asthma, hypersensitivity pneumonitis and other allergic disorders of the gastrointestinal tract needs to be clarified.

3. In AIDS, there is an increasing number of reports linking the HIV-1 virus and LDC (Belsito et al., 1982; Niedecken et al., 1987). Human epidermal LDC express CD4, HLA-DR antigens together with Fc-IgG receptors, which are all involved in the receptor binding of the HIV1 virus (Stingl et al., 1990). LDC may be a target cell for the HIV-1 virus. Stingl et al., (1990) reported a low density of epidermal LDC in patients suffering from AIDS. Damaging LDC by the virus will compromise their function to stimulate T-lymphocytes causing further immunodeficency. They also reported HIV-1-like particles on the surface of LDC and in the extracellular spaces. This was also supported by Zemelman et al., (1994) as they reported epidermal LDC bearing CD4 receptors, which make them targets for infection with HIV-1. As LDC can exfoliate through the epithelial surfaces, LDC infected with HIV in saliva, vaginal and rectal secretions may act as a vehicle to transmit the virus during sexual contact.

4. With transplantation, LDC can stimulate allogeneic and syngeneic reactions (Hammar, 1988). Odling et al., (1987) investigated the survival of skin grafts on animals treated with the chemical carcinogen (DMBA) to deplete LDC from the epidermis. Odling and co-workers reported longer survival of skin grafted on animals with depleted LDC in their skin. This was also supported by McMinn et al., (1990), reporting similar findings. Hill et al., (1994), used UV-B

irradiation to decrease corneal LDC number and reported improvement in corneal graft survival in rabbit. It may be possible to control rejection against a transplanted organ by pharmacological agents which modify the function of these antigen presenting cells.

5. Non-neoplastic diseases, LDC have been identified in about 60 human diseases, most being related to skin disorders. The majority of these reports was concerned with the identification of LDC rather than investigating their immunological role. Table 1.1 shows a list of such reports on LDC and human diseases.

1.9 Langerhans dendritic cells and human tumours

Basset et al., (1974) were first to report LDC in human tumours. They identified LDC by ultrastructural examination of cells in the bronchioalveolar lavage fluid of patients with bronchioalveolar carcinoma of the lung. The widespread distribution of LDC in human tumours was shown by Hammar et al., (1986), using S100 protein as a cytoplasmic marker for LDC together with ultrastructural tissue examinations. LDC were found in the following tumours- pulmonary adenocarcinoma, nasopharyngeal carcinoma, ovarian adenocarcinoma, gastric carcinoma, renal cell carcinoma, breast carcinoma, pancreatic carcinoma, thymoma, basal cell carcinoma, melanoma and in skin appendage neoplasms.

The density of LDC in actinic keratosis, Bowen's disease, keratoacanthoma, squamous cell carcinoma and basal cell carcinoma of

the skin was investigated by McArdle et al., (1986), who reported a high density of $S100^+$ LDC in all skin lesions except actinic keratosis. The increase in density of LDC in basal cell and in squamous cell carcinomas was supported by Missner et al., (1986).

Stene et al., (1988) investigated the density of Leu-6 (CD1a) positive LDC in various pigmented lesions of the skin and reported a decrease in the number of epidermal LDC in deeply invasive melanomas. They also reported normal LDC density in naevi, in early invasive melanomas and in metastatic cutaneous melanomas. Stene and co-workers, (1988) proposed that epidermal LDC may recognise a tumour associated antigen expressed by the neoplastic melanoma cells and presented to T-lymphocytes in the tissue and regional lymph nodes.

The density of LDC in human cervical intraepithelial neoplasia and wart viral infection was investigated by McArdle and Muller (1986). They observed a significant decrease in LDC number in cervical wart infection, possibly due to the effect of HPV on cervical LDC. They also found a marked increase of LDC density in cervical intra epithelial neoplasia (CIN). They proposed the decrease in the density of cervical LDC caused by HPV infection reduced the local cervical immunosurveillance and may promote the development of cervical cancer. Increase of LDC density in CIN has also been reported by Caorsi and Figueroa, (1986).

Bonilla-Musoles et al., (1987) investigated LDC distribution and their relationship to cervical cancer. They reported an increase in the HLA-DR positive LDC in squamous cell carcinoma of the cervix but did not find

Table 1.1 Identification of Langerhans dendritic cells in human diseases

Disease	Author/Year
Vitiligo	Birbeck et al.,(1961)
Psoriasis	Bos et al., (1986)
Urticaria pigmentosa	Johansson, (1985)
Granulomatous skin diseases	Aoki et al., (1987)
Keratoacanthoma	McArdle and Muller, (1986)
Allergic conjunctivitis	Takeuche et al., (1983)
Hypersensitivity pneumonitis	Hammar et al., (1986)
Pulmonary fibrosis	Basset et al., (1976)
Progressive systemic sclerosis	Andrews et al., (1986)
Connective tissue diseases	Goobar et al., (1986)
Sarcoidosis	Berman et al., (1986)
LDC granulomatosis (Histiocytosis X)	Hance et al., (1988)
Chronic liver diseases	Tobe et al., (1982)
AIDS	Stingl et al., (1990)
Cervical HPV	Vayrynen et al., (1984))
Behcet's disease	Kohn et al., (1984)
Fabry's disease	Le-Charpentier et al., (1978)
Omen's diseases	Ruco et al., (1985)

LDC in cervical adenocarcinoma. None of these studies correlated LDC density to the survival of patients. The association between high LDC number in SCC of the cervix and longer patients survival was also reported by Xie, (1990). Schenk, (1980) identified the ultrastructure of LDC in laryngeal carcinoma but no further investigations were reported.

Using S100 protein to identify LDC, the correlation between LDC density in human tumours and patient survival has been reported for lung tumours (Furukawa et al., (1984); nasopharyngeal carcinomas (Nomori et al., 1986); gastric carcinomas (Tsujitani et al., 1987); papillary thyroid tumours (Schroder et al., 1988); colorectal carcinomas (Ambe et al., 1989); oesophageal squamous cell carcinomas (Matsuda et al., 1990); laryngeal carcinoma (Rucci, et al., 1991); prostatic carcinoma (Bigotti, et al., 1991); cervical squamous cell carcinoma (Xie, 1990) and transitional cell carcinoma of the urinary bladder (Inoue, et al., 1994). They all reported longer survival of patients associated with high density of LDC in their tumours.

It is noteworthy that when Furukawa et al., (1985) investigated the distribution of $S100^+$ LDC in 40 patients with stage Ia lung adenocarcinoma; they reported a longer survival of patients with a high density of LDC in their tumours, although they correlated the density of LDC with tumour differentiation, they did not classify adenocarcinoma according to the cellular subtypes- ciliated, Clara, mucous producing and bronchioalveolar nor investigated LDC in SCC of the lung (Herrera et al., 1983 and Kawai et al., 1988).

Other workers have identified LDC in other human tumours, in pleomorphic adenoma of salivary gland (David, 1980), in non-Hodgkin's lymphoma (Daniel et al., 1985 and Neuman et al., 1986), in Hodgkin's lymphoma (Flint et al., 1987) and in leukemia (Kaiserling et al., 1988).

With regard to Hodgkin's lymphoma, Hsu and Hsu, (1990) identified an antigen, Mr70,000 (cell surface marker) expressed by both Reed-Sternberg cells of Hodgkin's lymphoma and the interdigitating reticulum cells of lymph nodes and spleen. The relationship between Hodgkin's lymphoma and LDC warrants further investigation.

Primary LDC tumours were reported in the skin by Kaiserling and Horny (1988) and by Strickler et al., (1990) and in the lymph nodes by Weiss et al., (1990) and by Yamakawa et al., (1992). The neoplastic diseases of LDC in peripheral blood, lymphoid tissue and solid organs warrants further investigation.

During 1993 many workers investigated the role of dendritic cells in tumours. Zeid and Muller, (1993), reported dendritic cells in human lung tumours are related to tumour type, cell differentiation and enhanced patients survival. Zeid and Muller, (1995) also reported the increase in dendritic cells in skin tumours in mice was associated with tumour regression. The anti-cancer activity of the bone marrow derived LDC has been investigated by Becker, (1993), Deutsch et al., (1993), Tsujitani, et al., (1993) and Austyn, (1993). While Becker (1993) reported high LDC density in primary human and experimental tumours was associated with tumour regression, Tsujitani, et al., (1993) demonstrated

that high density of LDC in regional lymph nodes to gastric carcinoma was associated with low incidence of nodal metastasis.

1.10 Langerhans dendritic cells in tobacco smoke induced pulmonary disorders

The role of antigen presenting LDC in lung pathology is incompletely understood. Nezelof et al., (1973) first reported LDC in the bronchio-alveolar lavage fluid of patients with pulmonary histiocytosis. LDC were reported in pulmonary fibrosis (Hammar et al., 1986), hypersensitivity pneumonitis (Hammar et al., 1986; Kawanami et al., 1987) and pulmonary histiocytosis (Nezelof et al., 1973)

The ultrastructural examination of Histiocytosis X cells in pulmonary histiocytosis showed that these cells contain the characteristic cytoplasmic marker of LDC, the BG. Since then pulmonary histiocytosis is called pulmonary Langerhans cell granulomatosis (Lieberman et al., 1980; Hance et al., 1988).

The effects of cigarette smoking on the bronchial epithelium and lung parenchyma have been documented in both human and animal models. Auerbach et al., (1957), investigated the bronchial epithelial tobacco smoke induced changes in the human lung and reported basal cell hyperplasia, stratification and squamous cell metaplasia of the bronchial epithelium as pre-cancerous changes in the lung. Wynder et al., (1961), applied tobacco condensate on the skin of various laboratory animals and reported skin papillomas and carcinomas after 8 to 12 months of

condensate application. Leuchtenberger et al., (1961), investigated the effect of tobacco smoke on the murine lung by exposing them for up to 28 months. They reported bronchitis, peribronchitis, atypical bronchial epithelial proliferation and occasional lung adenomas. Harris et al., (1974), exposed mice to a smoke:air mixture throughout their lives and reported alveogenic, adenocarcinomas and keratinized tumours in their lungs. The effect of tobacco smoke on the defence mechanisms of the respiratory tract was investigated by other workers (Ehrlich, 1966; Zarkower and Morges, 1972; Miller and Zarkower, 1974;). Holt et al., (1973), proposed that inhalation of tobacco smoke may damage local immunity and the immunosurveillance mechanisms of the respiratory tract causing recurrent respiratory infections and the development of some lung tumours. Thomas et al., (1978), investigated the effect of the vapour phase tobacco smoke on the phagocytic activity of macrophages and reported transient depression followed by enhancement of phagocytosis activity.

Nezelof et al., (1973) were the first to report LDC in pulmonary histiocytosis X, while Basset et al., (1974) were first to identify the ultrastructure of LDC in bronchioalveolar carcinoma. The effect of tobacco smoke on pulmonary LDC is unclear. There are only two reports to date in the literature reporting findings in this regard. Casolaro, (1988), reported accumulation of LDC in the epithelium of the lower respiratory tract of smokers. Soler et al., (1989), reported an increase in LDC and other related DC in smokers lung. As antigen presenting cells are capable of stimulating resting T-lymphocytes, their relation to other cells (alveolar macrophages, eosinophils, smooth muscle, plasma and

endothelial cells) in the pathogenesis tobacco induced lesions in the lung is yet to be clarified.

1.11 Analysis of tobacco smoke

More than 4270 chemical compounds have been identified in cigarette smoke (Dube and Green, 1982; Bos and Henderson, 1984). These chemical compounds include water, nicotine, polycyclic aromatic hydrocarbons (PAHs), N-heterocycles, nitrosamines, pesticides, organic acids, keto compounds, phenols and metals (Fresenius, 1985). Table 1.2 is a list of these compounds.

1.11.1 Carcinogens and co-carcinogens in tobacco smoke

Seventy one chemical compounds have been identified as carcinogens and co-carcinogens in tobacco smoke (Bos and Henderson, 1984). Hoffmann and Wynder, (1971) reported the chemical analysis of a neutral fraction and subfractions B and B1 of tobacco smoke. The most common co-carcinogens in this fraction were polycyclic aromatic hydrocarbons, benzo(a)pyrenes and catechol from the phenol group (Brunnemann et al., 1977). N-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone are the most powerful carcinogens formed by burning nicotine and other tobacco alkaloids nitrosamines (N-nitrosamines). Tobacco smoke condensate (TSC) was painted on the skin of mice, rats and Syrian golden hamsters to induce skin tumour formation (Hoffmann and Wynder, 1986).

Table 1.2 Chemical compounds found in tobacco (Fresenius, 1985)

group	chemical compounds	µg/cigarette
Polycyclic aromatic hydrocarbons (PAHs):		
	Benzo(a)pyrene	0.04
	Methylchrysene	0,02
	Dibenz(a,h)anthracene	0.004
	Benzo(b)fluoranthene	0.003
	Benzo(j)fluoronthene	0,006
N-Heterocucles:		
	Indol	1.4
	Skatol	4.3
	Carbazole	0.7
	Methylcarbazole	0.15
	Harman	13,00
	Nrthharman	4,5
	Dibenzo(c,g)carbazole	0.07
Nitrosamines:		
	Dimethylnitrosamine (mainstream)	1.7-97 ng/cigarette
	Dimethylnitrosamine (sidestream)	680-1770 ng/cigarette
Pesticides (Organic acids):		
	Acetic acid	205-620
	Benzoic acid	25-258
	Butyric acid	15-46
	Propionic acid	17-61
Keto compounds:		
	Acetone	27-562
	acetaldehyde	4-856
	Acrolein	1-87
	Aldehyde	0.9 -1.2
	Formaldehyde	2-7

Phenols:

Phenols (mainstream)	228
Phenols (sidestream)	603
o-Cresol	22
p-Cresol	18-40
pyrocatechol	61
hydroquinone	83
Resorcinol	8

Metals:

43 metals including, aluminium, antimony, arsenic, barium, lead, cadmium, caesium, cerium, chromium, iron, europium, gold, hafnium, cobalt, copper, lanthanium, lithium, lutetium, magnesium, manganese, molybdenum, nickel, polonium, mercury, radium, rubidium, samarium, scandium, selenium, silver, strontium, terbium, thorium, titanium, uranium, vanadium, bismuth, ytterbium, zinc, tin and zirconium have been identified in tobacco smoke.

Ruby et al., (1989) investigated the effect of benzo[a]pyrene and catechol on the epidermal LDC of mice and reported that both of these tobacco-derived carcinogens caused an increase in Ia^+ LDC density in the skin. They also reported abnormal LDC morphology, LDC appeared smaller than normal and with shorter dendrites. Following 24 weeks of BP application to the dorsal skin, 35% of mice developed skin tumours, 58% were squamous papilloma and 42% were squamous cell carcinoma. The BP treated skin showed hyporesponsiveness to a contact sensitizing agent, dinitrofluorobenzene. The impaired function of epidermal LDC may compromise the skin anti-tumour immunosurveillance system and allow tumour development.

1.11.2 Carcinogenesis of passive smoking

The mainstream tobacco smoke is generated during puffing the cigarette, which may not allow complete burning of the tobacco. The concentration of the chemical compounds in the mainstream tobacco smoke including carcinogens will be less than that of the sidestream tobacco smoke. The latter is generated by slow burning of the tobacco and will contain higher concentrations of chemicals compounds and carcinogens than that of the mainstream. Hoegg, (1972) reported that 80-90% of air pollution in closed spaces is caused by side stream tobacco smoke. Hirayama, (1981) investigated 91,540 non-smoking married women for 14 years and reported that wives of heavy smokers have a higher risk of developing lung cancer than wives of non-smokers. Bos et al., (1984) reported higher urinary output of metabolites relating to the chemical compounds in the tobacco smoke in men exposed to passive

smoking compared with the values before exposure. Later it was confirmed that the concentration of chemical compounds in the side stream tobacco smoke was 50-100% higher than in the mainstream tobacco smoke (Hoffmann and Wynder, 1986).

1.12 Summary

The bone marrow derived LDC are the best known potent antigen presenting cells and are capable of stimulating naive T-lymphocytes in organ associated lymphoid tissue and in the regional lymph nodes to initiate a specific immune response. They include the epidermal LDC, dermal indeterminate dendritic cells, veiled dendritic cells in the afferent lymphatics, interdigitating dendritic cells in lymph nodes and spleen, interstitial dendritic cells in the connective tissue of various body organs, and their precursors in the blood and in the bone marrow.

To date the effect of tobacco smoke on the density and function of the pulmonary antigen presenting dendritic cells and their role in tobacco smoke induced lung lesions and lung tumours have not been fully investigated.

The aims of this thesis were to study LDC in tobacco smoke related human lung cancer and to investigate the effect of passive tobacco smoke inhalation on murine pulmonary LDC. Further investigations e.g. the effect of crude tobacco smoke condensate on murine epidermal LDC during cutaneous carcinogenesis and investigating the $S100^+$ cells in the peripheral blood arose during the course of the study.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Specimens for human lung tumour study

Paraffin blocks of 130 lung tumours, 100 regional lymph nodes, 13 normal lung, 7 normal bronchial lymph nodes were obtained from the files of the Department of Anatomical Pathology, Royal Hobart Hospital. Three 3-4 μm sections were cut from each block, one for S100 staining, one for antibody control and the third for haematoxylin and eosin staining.

2.2 Clinical data

All data related to patients involved in this study were obtained from the files of the Medical Records Department, Royal Hobart Hospital.

2.3 Preparation of human tracheal and bronchial mucosal sheets

Four segments, 2 X 1 cm each of unfixed trachea and major bronchus from two autopsies were incubated in ethylene diamine tetraacetic acid solution (0.38 g EDTA in 50 ml PBS, pH 7.2; Serva, 11282). The incubation time and temperature were modifications of those suggested by Scaletta and MacCallum (1972). The mucosal segments were incubated for 3 hours at 37°C and overnight at 4°C. The tracheal and bronchial segments were treated with 50% ethanol for 30 minutes. The mucosal sheets were separated gently from the underlying cartilage, flattened on filter paper and processed for routine paraffin embedding; mucosal segments were blocked in paraffin as a flat mucosal sheet. The whole tracheal and

bronchial mucosa was tangentially and serially sectioned at 3 micron. A total of 159 sections was prepared for S100 and routine H&E staining.

2.4 Immunohistochemical staining of Langerhans dendritic cells in human materials

Rabbit antibody to bovine S100 protein (Dakopatts, Denmark, Z 311) was used to identify the dendritic cells in paraffin sections. The paraffin wax of the sections was first melted at 56°C for two hours, dried overnight at 37°C and then the paraffin wax was remelted at 56°C for 5 minutes before dewaxing in two changes of xylene, 5 minutes each. Sections were hydrated in descending concentrations of ethanol to water. Dewaxed sections were placed in prewarmed phosphate buffer saline (PBS), pH 7.4 at 37°C for 5 minutes. Enzyme digestion was then carried out by placing the sections in prewarmed 360 ml PBS, pH 7.4 containing 0.02 g of protease (Sigma No. 8038) and incubated at 37°C for 6 minutes. After washing the sections in running water for 5 minutes they were rinsed in 0.05 M Tris-HCl saline, pH 7.6 for 5 minutes. After drying, S100 antibody diluted 1:400 in diluent (1% bovine serum albumin, Sigma A 7030; and human serum group AB; CSL 1121001) was applied to the sections and incubated for 90 minutes at room temperature. Sections were washed in 0.05 Tris-HCl, pH 7.6 for 5 minutes. Sections were then incubated for 50 minutes at room temperature with the secondary antibody (biotinylated anti-rabbit immunoglobulin, Biogenex HK 326-UR) diluted 1:500 as for S100 antibody. After washing sections in 0.05 M Tris-HCl saline, they were incubated with alkaline phosphatase labelled streptavidin (Biogenex UK 321 UK) diluted 1:50 in Tris-HCl 0.05M pH 7.6. Sections were incubated at room temperature for 50 minutes. Sections were rinsed in 0.1 M Tris-HCl pH 8.2 after washing in 0.05 M Tris-HCl pH 7.6. The

phosphate in Tris buffer) and the chromagen Fast Red, (fast red TR salt and levamisol, Biogenex, USA, HK 182-5K) were applied to the sections and incubated at room temperature for 20 minutes and then counterstained in Mayers haematoxylin for 3 minutes after washing in deionised water for 5 minutes. Following washing in deionised water sections were mounted in crystal/mount (Biomeda, USA, M02), cleaned in xylene and finally mounted in Eukitt (Carl Zeiss, West Germany, 00 20 02).

2.5 Identification and counting of S100 positive Langerhans dendritic cells

The $S100^+$ Langerhans dendritic cells were identified by their red cytoplasm, indented nuclear membrane and prominent nucleolus. Chondrocytes of bronchial cartilage, myoepithelial cells of the serous glands in the lamina propria and nerve fibres are $S100^+$ in lung sections, but these cells were readily distinguished from the $S100^+$ dendritic cells.

$S100^+$ Langerhans dendritic cells (LDC) were counted in four high power fields (HPF, X 400) randomly selected for each section. These fields covered the tumour itself and zonal junction between the tumour and the surrounding lung parenchyma. The mean of the dendritic cell number in the four high power fields and the standard deviation were calculated for each case.

The relationship between the $S100^+$ LDC in each type of lung tumours and the density of tumour infiltrating lymphocytes (TIL) in and around tumours was recorded. The density of TIL was evaluated as mild, moderate and marked. Enumeration of $S100^+$ DC in lymph nodes was performed following the same methodology applied to lung sections.

2.6 Animals for tobacco smoke inhalation

BALB/c female mice 6-8 weeks old were obtained from the Central Animal House at the University of Tasmania after Ethics Committee approval. The 108 mice used for the tobacco smoke inhalation experiment were divided equally into two groups- experimental and controls. Control and experimental mice were maintained separately in groups of 6.

2.7 Experimental design of tobacco smoke inhalation

The experimental mice were exposed daily to the tobacco smoke of 8 cigarettes. Each cigarette needed 2 minutes to burn completely thus mice were exposed for a maximum of 16 minutes per day. The smoke:air ratio used in this study was 1:12; other researchers have used ratios of 1:7 (Leuchtenberg et al., 1960; Keast and Taylor; 1983; Wynder et al., 1985). The smoke:air ratio is vital to protect the mice from acute smoke toxicity and death. During the first week of the experiment, mice were exposed to the smoke of only one cigarette a day to acclimatise the mice to the much higher dose of 8 cigarettes during the second week. Six test and six control mice were killed by cervical dislocation at weeks 1 and 2 and then every 4 weeks. One lung from each mouse was snap frozen in liquid nitrogen and stored at -80°C for ZIO and anti-Ia immunoperoxidase staining. The other lung, together with the trachea and the bronchi, were perfused overnight in 10% formalin in PBS (pH 7.3) and processed for histological examination. At 28 weeks, and when the histological examination of the lungs of the test group showed positive tobacco induced changes, the remaining mice ceased to be exposed to further tobacco smoke. They were

maintained for a further 6 weeks to study the reversibility of the tobacco induced changes in the lung.

2.8 Tobacco smoke chamber

The tobacco smoking chamber (Fig. 2.1) was designed by the author N. A. Zeid. It consisted of three units, a smoke generating unit, a smoke exposure tent and an air outflow unit. The smoke generating unit was a 500 ml filter (Buchner flask, Selbys, Cat. No. 414644, FB500/3) connected by plastic tube to a central continuous source of air. The rate of air flow can be adjusted by a valve switch. A cigarette holder was fitted in the centre of the cover of the flask. The mouthpiece of the cigarette holder was connected by plastic tube to the smoke exposure tent. The central air flow was switched on before lighting the cigarette and firmly closing the flask. The air flow to the flask was adjusted to ensure the two minutes time needed for complete burning of the cigarette. The air in the flask together with the generated tobacco smoke flowed through the cigarette holder and plastic tube to the smoking tent. The amount of smoke generated from burning one untipped cigarette was about 350 ml (50 ml syringe was used to measure smoke from burning cigarette until completely burned). The smoke exposure tent was designed to provide the mice in the tent with environmental air similar to that of any enclosed place in the human situation. It consisted of a plastic tent and mouse cage. The tent was constructed from transparent plastic and supported by a steel frame. The tent was not sealed at the lower margin in order to provide continuity and similarity between the tent and the room environmental air. The tent was provided with a smoke inlet and safety smoke outlet connected to a central adjustable air suction. A standard animal cage was used provided with 74 holes at all sides, each hole about

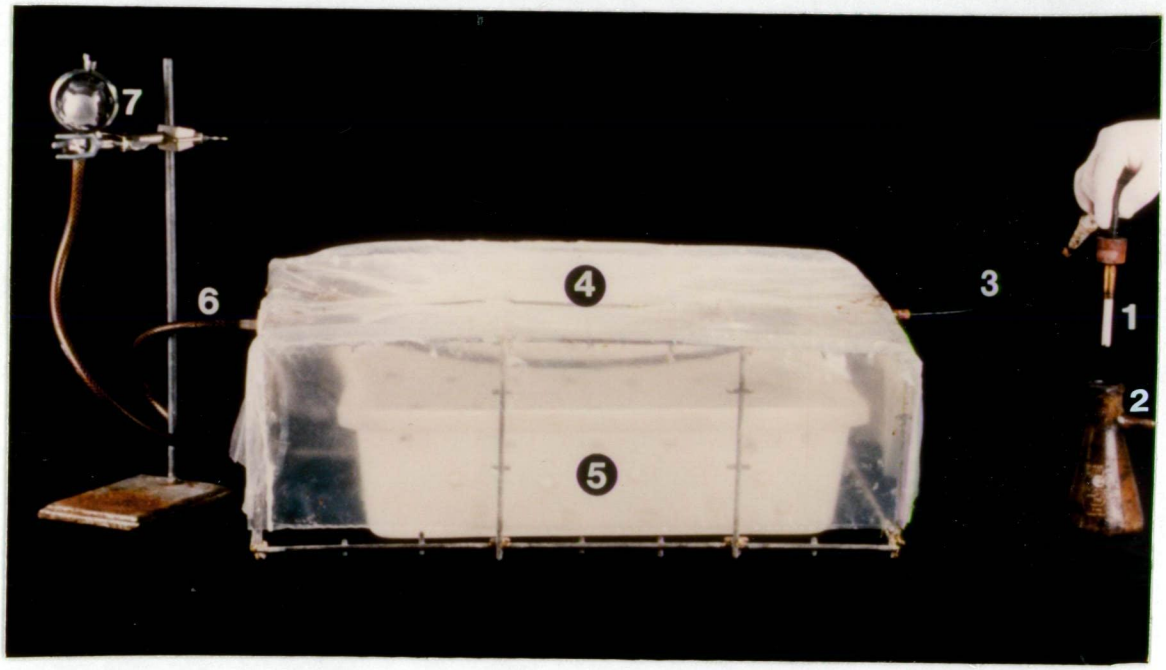


Figure 2.1 Tobacco smoke chamber consisting of smoke generating unit with a cigarette holder (1) connected by plastic tube (2) to a central continuous source of air. This was connected by plastic tube (3) to the smoke exposure tent (4) which covered the mouse cage (5). The air outflow unit was composed of a safety smoke outlet (6) and adjustable central suction (7).

1 cm in diameter. The cage measured 50 X 33 X 13 cm. The air outflow unit consisted of a safety smoke outlet connecting the tent to the adjustable central suction.

2.9 Tissue preparation of murine lung

Representative sections from the mice lung, bronchi and bronchial lymph nodes were processed and vacuum embedded in wax. Four μm sections were cut and stained with haematoxylin and eosin for light microscopy examination.

2.10 ZIO procedure to visualise Langerhans dendritic cells in mouse lungs

A modified ZIO procedure (Champy, 1913 & Rodriguez and Caorsi, 1987) was developed because of the nature of the lung. The ideal size and thickness of the lung tissue was 5 X 5 X 2 mm to ensure complete penetration of the fixative and staining solution. The frozen lung sections were fixed in V-7.4-ZIO solution (veronal sodium-HCl buffer solution-pH 7.4) at 4°C for 19 hours and then processed for routine paraffin embedding after washing in running tap water for 5 minutes. Five μm sections were cut, dewaxed in 2 changes of xylene, 5 minutes each and mounted directly in Eukitt mounting media.

2.11 Preparation of V 7.4-ZIO solution

A mixture of 2.4 g zinc powder (BDH Australia, 30596) and 1 g of iodine bisublimate (Ajax, Sydney-Melbourne, 70516) was added to 40 ml of 0.2 M 7.4 veronal sodium-HCl buffer. After shaking the mixture for ten minutes the solution was immediately filtered. The filtered solution was

mixed with 2% Osmium tetroxide (OsO_4, BDH Australia, 29422) at a ratio of 3:1. This solution was prepared fresh for each experiment.

2.12 Preparation of 0.2 M, 7.4 veronal sodium-HCl buffer

N/10 hydrochloric acid (HCl): 8.5 ml of concentrated HCl per one litre of distilled water.

Veronal acetate solution: Sodium acetate crystals, 19.4 g and 29.4 g of sodium diethyl barbiturate (Veronal) were dissolved in distilled water to make up to 1 litre. To prepare 0.2 M, 7.4 veronal sodium-HCl buffer solution, 5 ml of N/10 HCl, 5 ml veronal acetate solution was added to 15 ml of distilled water.

2.13 V-7.4-ZIO staining of mouse lungs for electron microscopy

Modifications of the procedure of Rodriguez and Caorsi, (1978) were used. Lung sections 25 μm thick, were cut from paraffin blocks of lung tissue which had already been incubated with V-7.4-ZIO solution. They were dehydrated in ascending concentrations of ethanol (30; 75; 90; 95; 100%), 20 minutes each and embedded in Epon resin (Procrue 812, 11.7 g; Dodecenyl succinie, 3.3 g; Nadic methyl anhydride, 8.4 g; Tri-dimethylaminoethyl phenol, 0.3 g).

2.14 Immunoperoxidase staining to visualise Langerhans dendritic cells in mouse lungs

Five μm acetone-fixed frozen sections of lung tissue were mounted on potassium-dichromate-gelatine coated slides, dried for 2 hours, incubated

with mouse monoclonal anti-Ia (MKD6, Kappler et al., 1981) for 90 minutes at room temperature, washed 3 times in phosphate-buffered saline (PBS, pH 7.4) over 20 minutes, and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin (HRPO-anti-Ig, 1:80; Dakopatts, Denmark). Sections were washed in PBS, three times over 20 minutes. The Ia^+ cells were identified by incubating the sections with diaminobenzidine (DAB, Sigma- D5637, 0.5 mg/ml of PBS containing 0.02% H_2O_2, pH 7.4) for ten minutes. Sections were washed with running water for 10 minutes and counterstained with Mayers haematoxylin. The washed sections were mounted in glycerol gelatin (Sigma, 86F-6098) for microscopic examination.

2.15 Enumeration of ZIO positive LDC in lung sections

The number of ZIO^+ LDC in four HPF (X400) was calculated. The mean and the standard deviation were statistically analysed for groups of mice assessed.

2.16 Animals for tobacco smoke condensate (TSC) cutaneous experiment

Two hundred and four BALB/c female mice 6-8 weeks old were obtained from the Central Animal House at the University of Tasmania after Ethics Committee approval. One group of mice (48) was used to assess the function of LDC by inducing a contact hypersensitivity reaction, while the remaining 156 mice were divided into 2 groups of 78 mice each, experimental and control. Control mice were treated with olive oil and acetone, while the experimental mice were treated with TSC in acetone and olive oil.

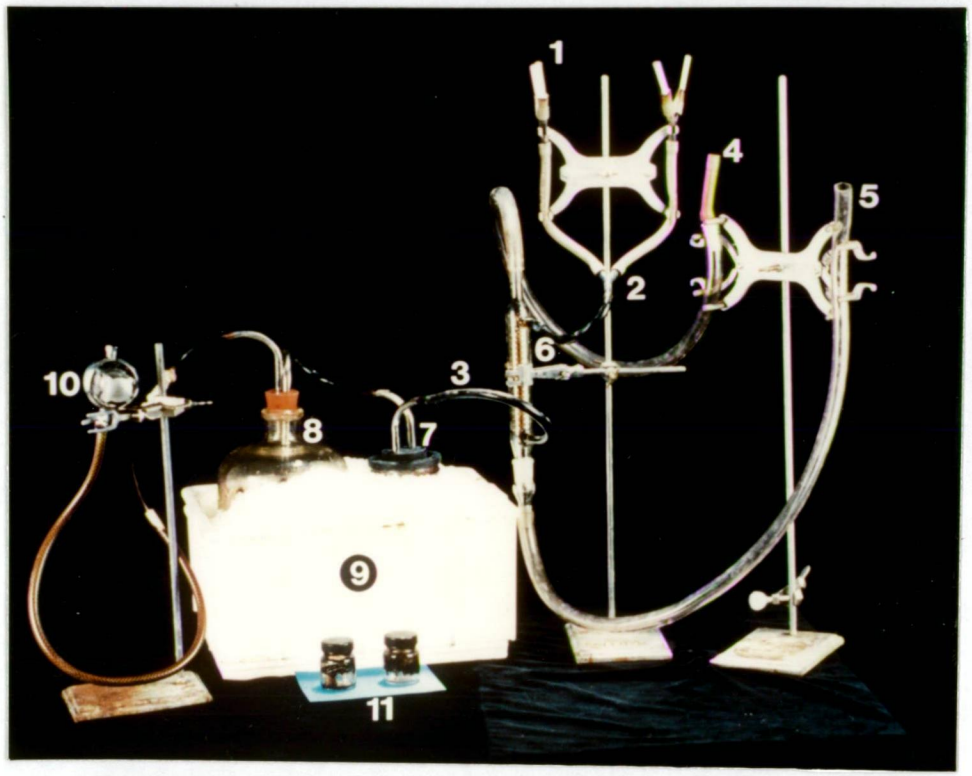


Figure 2.2 Unit used to prepare the tobacco smoke condensate. It consists of the following components:
Cigarette burning unit 1; 2.
Smoke condensation unit, 4; 5; 6.
Cold trap unit, 3; 7; 8; 9.
Suction unit, 10.

2.17 Preparation of tobacco smoke condensate

The TSC was prepared (Fig. 2.2) by condensation of cigarette smoke by a cold trap (Bentley and Burgan, 1961). TSC was collected in jars and the water content evaporated by placing the jars, after gradual warming, in a water bath adjusted at 100°C. TSC was then dissolved in an equal volume of acetone. Each 550 cigarettes gave 55 g (35 ml) of condensate. After dilution with an equal volume of acetone, the total amount of diluted condensate was 70 ml.

2.18 Experimental design of TSC cutaneous experiment

After shaving the dorsal skin of control mice, this group was treated with 200 µl of olive oil and acetone (1:1). The skin of the experimental mice was prepared as for the control, and treated with 100 µl of olive oil and 100 µl of tobacco smoke condensate in acetone (1:1). The treatment was applied every second day including the weekends. At 35 weeks TSC treatment was stopped, and the mice were kept for further 10 weeks to observe changes in tumour size and relationship to LDC numbers. Six mice from both groups, control and test were killed by cervical dislocation at 3 days, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 45 weeks. Skin sections were stained with haematoxylin and eosin and immunoperoxidase anti-Ia staining to visualise and count LDC in the prepared epidermal sheets.

2.19 Preparation of epidermal sheets

The method of Baker et al., (1983) was used to prepare epidermal sheets. The dorsal skin was shaved using electrical clippers and then depilated with a thioglycollate cream (Veet, Reckitt and Colman, UK). Adhesive

cellophane tape was then applied to the dry skin, the skin was removed and incubated at 37°C for 3 hours in tetra-sodium ethylenediamine tetraacetic acid solution (0.38 g EDTA in 50 ml PBS, pH 7.4; Serva, 11282). Using fine forceps the epidermis was separated from the dermis and stained with anti-Ia to demonstrate epidermal LDC.

2.20 Immunoperoxidase anti-Ia staining of LDC in epidermal sheets

Epidermal sheets were fixed in acetone at room temperature for 20-30 minutes and then incubated overnight at 4°C with mouse monoclonal anti-Ia (MKD6). After washing the sheets in PBS for 20-30 minutes, they were incubated at room temperature for 2 hours with horse peroxidase-conjugated rabbit anti-mouse immunoglobulin (HRPO-anti-Ig, Dakopatts, Denmark), diluted 1:80 in PBS, pH 7.4. Epidermal sheets were washed again in PBS for 30 minutes and incubated for 10 minutes with diaminobenzidine (DAB; Sigma; 0.5 mg/ml of PBS containing 0.02% H_2O_2, 7.4). The epidermal sheets were mounted in glycerol gelatin after washing in tap water.

The Ia^+ LDC were identified in the epidermal sheets as brown dendritic cells with various numbers of dendrites extending from the cell body.

2.21 Immunoperoxidase anti-Ia staining of LDC in tumour sections

Eight µm frozen sections of skin tumours were fixed in acetone for 20-30 minutes, washed three times in PBS over 20-30 minutes, incubated with anti-Ia for 30 minutes at room temperature, washed in PBS as before and incubated with HRPO-anti-Ig for 30 minutes at room temperature. Sections were again washed in PBS before incubation with DAB at room

temperature (Ruby et al., 1989) for 10 minutes and counterstained in Mayers haematoxylin after washing in running tap water for 5 minutes. Finally section were mounted in glycerol gelatin.

2.22 Enumeration of Ia positive LDC in epidermal sheets

LDC were assessed as the number of Ia^+ LDC/mm^2 and the mean standard deviation was calculated per group of mice.

2.23 Histology of murine skin

Skin sections of mouse tumours and normal control skin were processed for routine haematoxylin & eosin staining.

2.24 Contact hypersensitivity assessment

The function of LDC was assessed by inducing a contact hypersensitivity reaction at the 2nd and the 6th weeks, after commencing treatment with TSC. This was performed by applying TSC in a 1:1 solution of acetone:olive oil and the control mice acetone:olive oil alone. The dorsal skin of the test mice was initially sensitised with 10 µl of a 1% solution of 2,4,6-trinitrochlorobenzene (TNCP; Tokyo-Kasei, FCV61). The right ear of both control and test mice was challenged with 10 µl of a 1% TNCB solution 5 days after sensitisation. The thickness of the right and the left (unchallenged) ear was measured 24 and 48 hours later using an engineers spring loaded micrometer. The percent increase in ear swelling was calculated according to the following formula:

$$\% \text{ increase} = \frac{\text{thickness of right ear - thickness of left ear}}{\text{thickness of left ear}} \times 100$$

The average percent increase and the standard deviation was calculated for each group of mice.

2.25 Blood specimens

Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml of venous blood from each of 100 normal subjects. The heparinised blood was diluted 1:1 with PBS and layered over 9 ml of Histopaque-1077 (Sigma, USA) in a 30 ml centrifuge tube. After centrifugation at 2000 rpm (600g) for 30 minutes, PBMC were harvested from the upper interface and washed for 10 minutes in PBS at 1500 rpm (350g) and then twice at 1000 rpm (150g). The mononuclear cells were resuspended in 1 ml of PBS and counted using a Neubauer improved haemocytometer. Cell viability was assessed on the bases of the ability of live cells to exclude eosin Y dye.

2.26 Cytospins

PBMC were diluted in PBS-FCS (PBS containing 10% fetal calf serum-CSL, Melbourne) to a final concentration of 3 X 10^5 cells/ml. Two hundred µl of the cell suspension was centrifuged at 1100 rpm (100g) for 2 minutes. The cells on the slide were marked with a diamond pencil and dried overnight before staining.

2.27 Buffy coats

The anticoagulated blood was centrifuged at 1600 rpm (400g) for 15 minutes in Westergren tubes. The buffy coat layer containing WBC was

harvested with a pasteur pipette. A drop of the cell suspension was smeared on slide with the edge of another slide.

2.28 Metrizamide enrichment of LDC from the peripheral blood

The method of Knight et al., (1986) was adopted to isolate dendritic cells from peripheral blood. Blood was diluted 1:1 with PBS and 30 ml layered over 20 ml Ficoll/Paque (Pharmacia, Code No. 17-0840-02) and centrifuged for 30 minutes at 750 g. Peripheral blood mononuclear cells (PBMC) were collected from the interface and washed with PBS for 5 minutes. Thirty ml of PBS containing the PBMC was layered over 5-10 ml 14.5% metrizamide (Nycomed, 2344) added to 100 ml RPM1 and then centrifuged for 30 minutes at 750 g. Dendritic cells were collected from the interface washed with PBS, and cytospins were prepared for immunocytochemical staining.

2.29 Immunocytochemical staining of blood cells

Buffy coats and cytospins of the PBMC and metrizamide enriched LDC were fixed for 90 seconds in a mixture of acetone (190 ml), methanol (190 ml) and 40% formaldehyde (20 ml). Blood cells were stained using rabbit antibody to bovine S100 protein (Dakopatts, Denmark, Z 311) using the protocol previously described (Section 2.4). Monoclonal mouse anti-human T-cell- CD3 (Pan T cell, Becton Dickinson, USA; Lot No. 437), CD4 (helper/inducer T cell, combination of anti-Leu 3a + anti-Leu 3b, Becton Dickinson, USA; NO511), CD8 (suppressor/cytotoxic T cell, anti-Leu 2a, Becton Dickinson, USA; M1106), anti-human monocytes- CD14 (monocytes, MY4, Coulter Immunobiology, USA, 6390124), anti-leucocyte common antigen, (HLe-1, CD45, Becton Dickinson, USA; NC048) and

CD1a (LDC and thymocytes, Becton Dickinson, USA; 437) were used to characterise blood cells. $S100^+$ cells, lymphocytes and monocytes numbers were expressed as the number of cells $x10^9$ per litre of blood.

2.30 Double immunocytochemical labelling of PBMC and DC

Cytospins were fixed in acetone:methanol:formaldehyde for 90 seconds. To suppress endogenous peroxidase, the cytospins were incubated in 0.05M Tris-HCl containing 0.3% H_2O_2. Cytospins were then incubated with antibody to the S100 protein, followed by incubation with peroxidase conjugated swine anti-rabbit, and then with peroxidase anti-peroxidase. At this stage, cytospins were incubated with the diaminobenzidine (DAB, Sigma- D5637) substrate to localise the S100 protein. The second surface antigen was demonstrated using an indirect alkaline phosphatase technique. Cytospins were incubated with mouse anti-human surface antibody (CD1, CD3, CD4, CD8, CD14, CD45) and then incubated with alkaline phosphatase conjugated sheep anti-mouse antibody. The reaction colour for the second antigen was developed after incubating the cytospins with Fast Blue substrate solution (naphthol; N,N dimethyl formamide; Tris buffer; levamisol; Fast blue TR salt). Slides were covered with crystal mount and mounted in Eukitt.

2.31 Phagocytic assay of the S100 positive cells in the peripheral blood

Polystyrene latex beads (diameter 1.16 µm; Polysciences Inc., USA) were diluted, 2 drops in 10 ml PBS. Ten µl of the latex beads, 100 µl of PBMC at a concentration of 1 X 10^7/ml and 10 µl of foetal calf serum (FCS) were added to a small tube, vortexed gently and incubated at 37°C for 30 minutes. This was centrifuged at 2000 rpm (600g) for 5 minutes and the

supernatant discarded. The cells were washed twice in PBS-azide (PBS containing 0.1% sodium azide, BDH, England) at 600g for 5 minutes. The cells were then resuspended at 3 X 10^5 /ml, cytospins were prepared and stained for S100 protein.

2.32 Adherent and non-adherent PBMC

Peripheral blood mononuclear cells (PBMC) were resuspended in RPMI (Flow Laboratories, Scotland) supplemented with 5% FCS solution (RPMI-5%), at a concentration of 2 X 10^7 cells/ml. One ml of this suspension was placed in a 35 mm petri dish and incubated for 1 hour at 37°C in 5% CO_2 in air in a humidified incubator. After 1 hour the dish was washed gently 5 times with pre-warmed (37°C) PBS to remove the non-adherent cell fraction. These cells were first washed gently from the dish using pre-warmed PBS, cells were counted and the prepared cytospins were stained for S100 protein. To collect the adherent cells, 2 ml of RPMI-10% FCS containing 0.67% EDTA was added to the petri dish and incubated at 4 °C for 10 minutes. The dish was washed by flushing RPMI-10% FCS-0.67% EDTA over the surface with a pasteur pipette. The adherent cells were washed using PBS solution, cytospins were prepared and stained for S100 protein.

2.33 Olympus CUE-2 image analysis system

Using the image analysis system (Olympus CUE-2, versuion 4), the perimeter, volume, width and length of the $S100^+$ cells (n=75 cells) in peripheral blood was recorded and compared with that of the $CD3^+$ lymphocytes (n=55 cells) and $CD14^+$ monocytes (n=12 cells). The measured standard deviations were calculated.

2.34 Micrometer eyepiece

Using the micrometer eyepiece (Olympus OSM-D4), the perimeter, diameter and volume of $S100^+$ cells (n= 200) in peripheral blood was recorded and compared with that of the $CD3^+$ lymphocytes (n= 401 cells) and $CD14^+$ monocytes (n= 399 cells). The means and standard deviations were calculated.

2.35 Immunoelectron microscopy examination of PBMC $S100^+$ cells

The method described by Takahashi et al., (1981) was used for the immunoelectron microscopy examination of the $S100^+$ cells in blood. PBMC were fixed for 10 minutes in 2% paraformaldehyde containing 0.25% glutaraldehyde (Serva, Germany) solution, then washed in PBS. The cells were incubated with rabbit anti-cow S100 (1/300) for 2 hours. After washing in PBS, cells were incubated with peroxidase conjugated swine anti-rabbit for 1 hour. After washing in PBS, cells were incubated for 30 minutes with DAB (20 mg in 100 ml Tris-HCl). Cells were washed and incubated for 6 minutes in DAB solution containing H_2O_2. The cells fixedin 1% OsO_4 for 30 minutes and processed for routine electron microscopy. The negative control was treated as above except normal rabbit serum was substiuted for the primary anti-S100 antibody.

2.36 Specimens of normal spleens and bone marrow

Three 3-4 μm sections were cut (S100, control and H&E staining) from each paraffin blocks of 8 normal bone marrow and 10 normal spleen were obtained from the files of the Department of Anatomical Pathology, Royal Hobart Hospital.

2.37 Statistics

Statistics were performed using non-parametric Wilcoxon test, Bonferroni multiple-comparison test, Student's two-tailed, unpaired t-test and Scheffe's test. Logarithmic values were used when the range of values was not normally distributed.

Foot note

Selection of immunohistochemical staining to identify Langerhans dendritic cells in human and murine material

Selection of the type of immunohistochemical staining for specimens was based on the following:.

1. For human material S100 was used to identify Langerhans cells since formalin fixed material can be examined by this technique.

2. For murine experiments, as antibodies to S100 do not stain dendritic cells in this species, immunoperoxidase anti-Ia staining was used.

3. For electron microscopy of murine DC, the ZIO procedure allowed visualisation of Langerhans dendritic cells in mouse lungs. Anti-Ia could not be used for this.

CHAPTER 3

LANGERHANS DENDRITIC CELLS AND HUMAN LUNG TUMOURS

3.1 INTRODUCTION

Langerhans dendritic cells were first reported in human tumours (cervix) by Younes et al., (1968). Since then LDC have been reported in other tumours; bronchioalveolar tumour of lung (Basset et al., 1974), laryngeal carcinoma (Schenk, 1980; Rucci et al., 1991), pleomorphic adenoma of salivary gland (David and Buchner, 1980), skin tumours (Gatter et al., 1983; McArdle et al., 1986), lung (Furukawa, et al., 1981, 1984; Fox et al., 1989), breast and thymoma (Hammar et al., 1986), oesophageal (Matsuda et al., 1990), gastric tumours (Tsujitani et al., 1987), papillary thyroid carcinoma (Schroder et al., 1988), colorectal carcinoma (Ambe et al., 1989), nasopharyngeal carcinoma (Nomori et al., 1986), prostatic carcinoma (Bigotti, et al., 1991) and transitional cell carcinoma of the urinary bladder (Inoue et al., 1994). S100 protein was used as a marker for LDC in most of the above reports. Hammar et al, (1986) reported Birbeck granules in all the $S100^+$ dendritic cells in lung bronchioalveolar carcinoma and adenocarcinoma, nasopharyngeal cancer, ovarian malignant tumours, breast carcinomas, thymoma and in malignant histiocytosis.

Basset and colleagues (1974) first reported dendritic cells which were similar in ultrastructural features to LDC in the bronchioalveolar lavage fluid of patients with primary alveolar carcinoma of the lung. Since then Kawanami et al., (1981; 1987) and Richard et al., (1987) have described LDC with characteristic features in normal human lung. Sertl et al., (1986) and

Holt and Schon-Hegrad, (1988) have also reported MHC-class II positive dendritic cells in the bronchial epithelium and in the alveolar septum of normal lungs of mice, human and rats.

In human lung tumours, there are conflicting reports on LDC and prognosis. While Furukawa et al. (1981; 1984) associated longer survival with marked infiltration of LDC in stage 1a adenocarcinomas of the lung, Fox et al., (1989) suggested that a high number of LDC in lung tumours was associated with a worse prognosis. Fox and co-workers (1989) reported the presence of CD1 positive LDC mainly in squamous cell carcinoma and adenocarcinoma of the lung, while Hammar et al., (1986) reported that only 17% of squamous cell carcinomas contain LDC.

None of the above reports investigated the relationship between LDC, cellular subtype of tumour, tumour differentiation, tumour infiltrating lymphocytes (TIL) and patient survival.

The aims of this study were to:

1. Investigate LDC in normal mucosa of trachea, bronchi, bronchioles, lung and in normal trachio-bronchial lymph nodes regional to normal lung.
2. Assess the density of pulmonary LDC in various cellular subtypes of human lung cancer and its relationship to tumour differentiation.
3. Study the relationship between the density of LDC and tumour infiltrating lymphocytes.

4. Examine the relationship between the density of LDC in lymph nodes regional to lung tumours and the immunological activity of these nodes.
5. Investigate the effect of LDC in lung tumours on the survival of patients.

3.2 MATERIALS AND METHODS

3.2.1 Specimens for human lung tumours

130 lung tumours, 100 regional lymph nodes, 13 normal lung and 7 normal bronchial lymph nodes were used in this study, as described in Section 2.1.

3.2.2 Clinical data

All clinical data were obtained from the files of the Medical Records Department, Royal Hobart Hospital (Section 2.2).

3.2.3 Preparation of the human tracheal and bronchial mucosal sheets

Four segments, 2 x 1 cm each of unfixed trachea and major bronchus from two autopsies were to prepare the mucosal sheets (Section 2.3). The whole tracheal and bronchial mucosa was tangentially and serially sectioned at 3 micron. A total of 159 sections was prepared for S100 and routine H&E staining.

3.2.4 Immunohistochemical staining of Langerhans dendritic cells in human materials

Rabbit antibody to bovine S100 protein (Dakopatts, Denmark, Z 311) was used to identify the Langerhans dendritic cells in paraffin sections as described in Section 2.4.

3.2.5 Identification and counting of the S100 positive Langerhans dendritic cells

$S100^+$ Langerhans dendritic cells (LDC) were counted in four high power fields (HPF, X 400) randomly selected for each section and as previously desribed in Section 2.5. The density of TIL was evaluated as mild, moderate and marked. Enumeration of $S100^+$ DC in lymph nodes was performed following the same methodology applied to lung sections.

3.3 RESULTS

3.3.1 Density of S100 positive dendritic cells in normal tracheal, bronchial mucosa and lung

$S100^+$ LDC showing red cytoplasm, indented nucleus and dendritic cell membrane (Fig. 3.1) were rarely found in the intra epithelial component of the bronchial tree (Fig. 3.2). They were commonly seen in the lamina propria of the tracheal and bronchial mucosa close to the perivascular lymphoid tissue. An average of two $S100^+$ LDC were present in both tracheal and bronchial sections in every 3 HPF. LDC were observed in the alveolar septum of all sections of normal lung, but in various densities. Occasionally LDC were found in the alveolar spaces (Fig. 3.3).

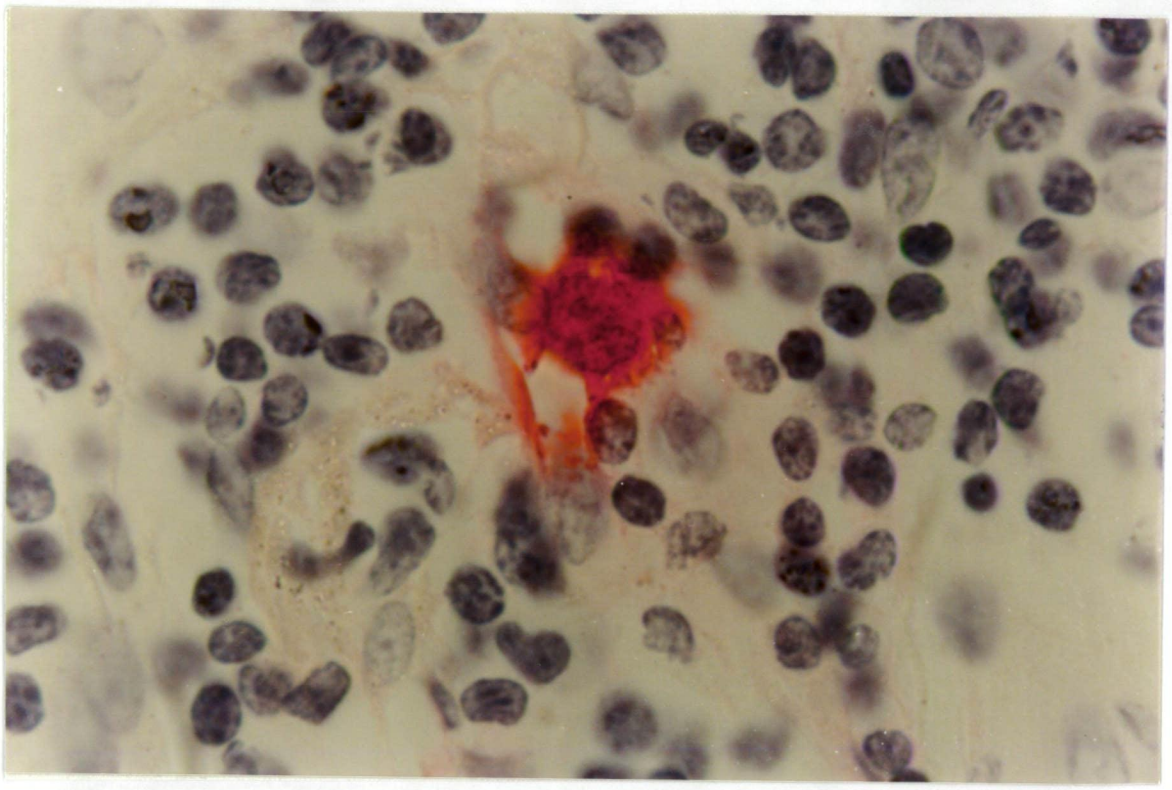


Figure 3.1 S100 positive Langerhans dendritic cell (LDC) shows red cytoplasm and indented nucleus
(S100 Immunohistochemical Technique X 400)

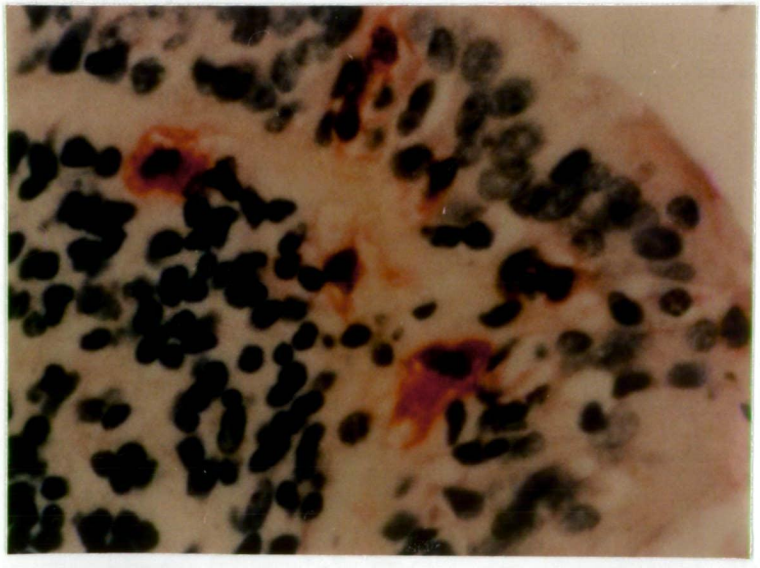


Figure 3.2 S100 positive Langerhans dendriticells in the bronchial epithelium and related lymphoid tissue
(S100 Immunohistochemical Technique X 250)

The mean of $S100^+$ LDC count in four HPF was 6 ± 4.9 cells in normal lung.

3.3.2 Density of $S100^+$ Langerhans dendritic cells in lung tumours

$S100^+$ LDC were found in direct contact with TIL and tumour cells in all varieties of lung tumours, but in various densities. Bronchioalveolar (Fig. 3.4), well and moderately differentiated squamous cell carcinomas (Fig. 3.5) showed the highest density of these cells within tumours. Small cell lung cancer, mucous producing adenocarcinomas and poorly differentiated squamous cell carcinomas contained the lowest number of $S100^+$ LDC. The number of $S100^+$ LDC related not only to tumour cell type, but also to tumour differentiation. While well and moderately differentiated squamous cell carcinomas contained 42.6 ± 21.2 LDC/HPF and 37.4 ± 10.8 LDC/HPF respectively, poorly differentiated squamous cell carcinoma contained only 13.2 ± 0.8 LDC/HPF. Table 3.1 summarises these results.

3.3.3 Langerhans dendritic cell morphology in lung tumours

The number of dendrites extending from LDC in relation to the histological variety of the lung tumours was examined. LDC were divided into two groups according to the number of dendrites extending from the cell body; LDC with more than 3 dendrites; LDC with less than 3 dendrites. LDC with more than three dendrites were found in 77.8% of bronchioalveolar carcinomas, 67.4% of well and moderately differentiated squamous cell carcinomas, in 34.7% of all varieties of adenocarcinomas

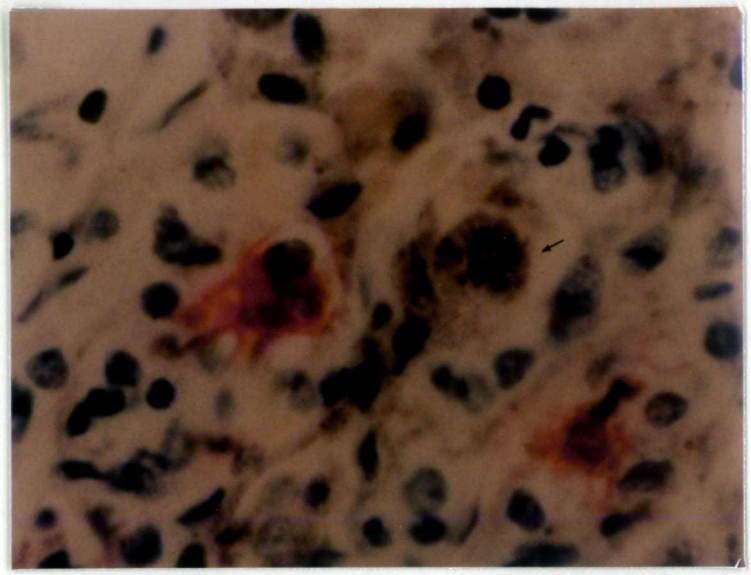


Figure 3.3 S100 positive Langerhans dendritic cell in the alveolar spaces. Alveolar macrophages (arrow) are negative for S100 protein (S100 Immunohistochemical Technique X 400)

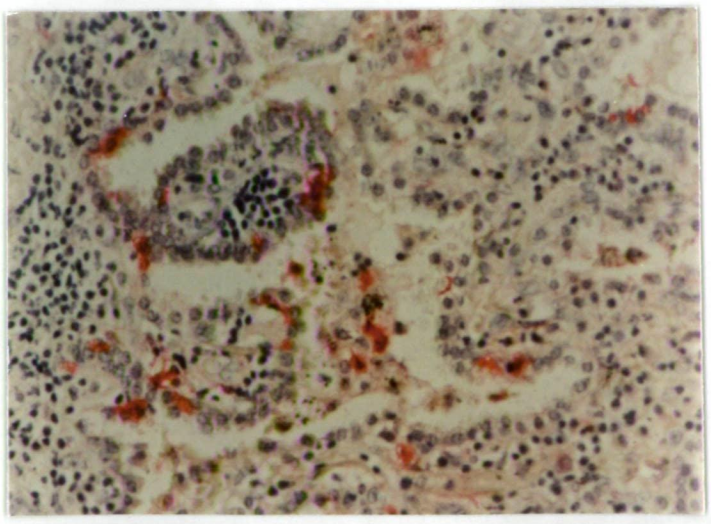


Figure 3.4 S100 positive Langerhans dendritic cells in bronchioalveolar carcinoma (S100 Immunohistochemical Technique X 250)

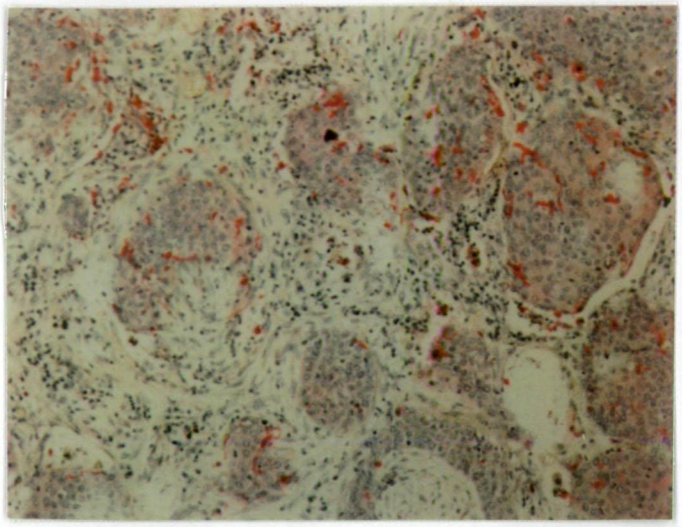


Figure 3.5 S100 positive Langerhans cells in well differentiated squamous cell carcinoma (S100 Immunohistochemical Technique X 100)

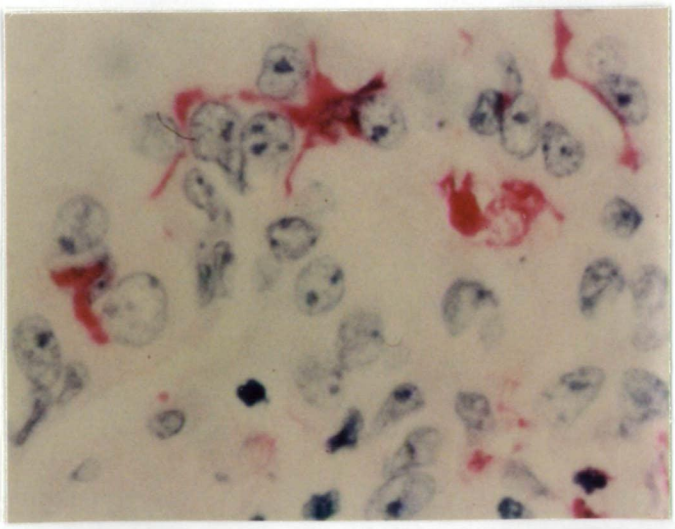


Figure 3.6 Direct contact between S100 positive Langerhans dendritic cells and adenocarcinoma tumour cells
(S100 Immunohistochemical Technique X 400)

Table 3.1 S100$^+$ LDC in various histological types of lung tumours

Histology	No. of cases	Mean of S100$^+$ LDC
1. Bronchioalveolar	16	57.8 ± 26.5
2. Well differentiated squamous cell carcinoma	16	42.6 ± 21.2
3. Moderately differentiated squamous cell carcinoma	14	37.4 ± 10.8
4. Poorly differentiated squamous cell carcinoma	38	13.2 ± 0.8
5. Nonciliated & Clara cell adenocarcinomas	18	25.1 ± 11.4
6. Ciliated & mucous producing adenocarcinomas	14	12.4 ± 5.7
7. Adenosquamous	4	24.25 ± 8.2
8. Small cell lung cancer	10	4.7 ± 3.8
Normal lung	13	6 ± 4.85

Using the non-parametric, Wilcoxon test, 1 versus 4, 5, 6 and 8 was significantly different, $P < 0.0005$. 2 versus 4, 5, 6 and 8 also significantly different, $P < 0.0001$, 0.0125, 0.0001 and 0.0003 respectively.

and in 32.6% of poorly differentiated squamous cell carcinomas. Dendritic cells with less than 3 dendrites were more commonly seen in the majority of small cell lung cancer and poorly differentiated squamous cell carcinoma.

3.3.4 Contact between S100+ Langerhans dendritic cells and lung tumour cells

Direct contact between LDC and tumour cells (Fig. 3.6) was consistently present in all bronchioalveolar carcinomas, in all well and moderately differentiated squamous cell carcinomas and in 81.3% of all varieties of adenocarcinomas. Contact between LDC and tumour cells was rarely observed in poorly differentiated squamous cell carcinomas and small cell lung cancers, although LDC were occasionally detected in these lesions.

3.3.5 Contact between S100+ Langerhans dendritic cells and TIL in lung tumours

Direct contact between LDC and TIL were found in all varieties of lung tumours (Fig. 3.7). In general there was a proportional relationship between the number of LDC and the density of TIL in and around various lung tumours. The density of TIL was marked in bronchioalveolar, and in both well and moderately differentiated squamous cell carcinomas. The density of TIL was moderate in other varieties of adenocarcinomas and mild in both poorly differentiated squamous cell and small cell lung cancer.

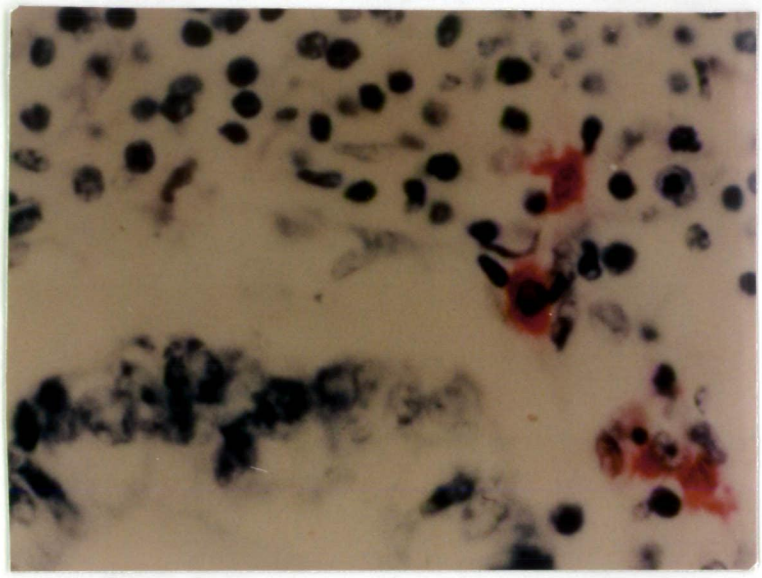


Figure 3.7 Contact between S100 positive dendritic cells and TIL
(S100 Immunohistochemical Technique X 400)

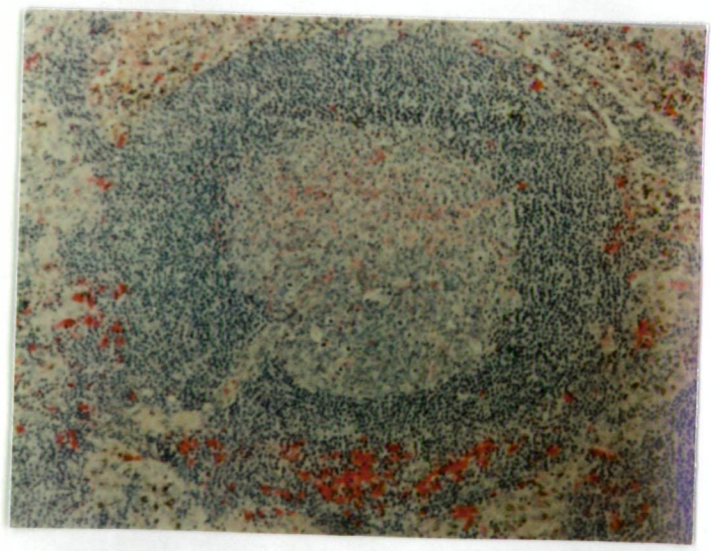


Figure 3.8 S100 positive dendritic cells in the T-zone of lymph node with active germinal centre
(S100 Immunohistochemical Technique X 250)

Table 3.2 S100+ LDC in normal regional lymph nodes

Histology of LN	No.	Mean of S100+ LDC/HPF	SD
Lymphocytic predominance	27	69.3	28.9 (a)
Active germinal centre	46	69.7	28.4 (a)
With metastasis	16	69.9	43.9 (a)
Unstimulated	33	24.8	13.4 (b)
Normal lymph nodes	7	35.0	10.3 (b)

Based on the Bonferroni multiple-comparison test, the number of S100+ LDC in lymph nodes with the same letter were not significantly different at the 5% level, while both groups (A) and (B) were significantly different.

Table 3.3 Lung tumours with the highest and lowest number of $S100^+$ LDC

	Highest No. of LDC	Lowest No. of LDC
1. No. of cases	27	41
2. $S100^+$ LDC/HPF	69.8 ± 19.4	6.55 ± 3.5
3. Density of TIL	Marked	Scanty
4. Average tumours size (cm)	3.5 ± 1.4*	4.7 ± 3.3*
5. LN Metastasis	33.3 %	34.3 %
6. M:F Ratio	3:1	3:1
7. Cigarettes/day	28.7 ± 15.4	27.6 ± 17.2
8. Smoking years	37.2 ± 10.3	36.3 ± 13.3
9. No. of Dead	8 (29.6%)	28 (68.6 %)
10. Survival (Months) since diagnosis	68.5 ± 32.9	8.3 ± 7.7
11. Histological typing	DSCC 59.3 % BA 33.3 % ADENO 7.4 %	PDSCC 55.2 % ADENO 31.0 % SCLC 13.8 %

DSCC = well and moderately differentiated squamous cell carcinoma
PDSCC = poorly differentiated squamous cell carcinoma
BA = bronchioalveolar carcinoma
ADENO = adenocarcinoma
SCLC = small cell lung carcinoma
* Maximum diameter of tumour

3.3.6 Central tumour necrosis, S100+ Langerhans dendritic cell number and TIL density in lung tumours

Central tumour necrosis was observed in lung tumours with a high number of LDC and marked density of TIL. Marked central tumour necrosis was found in 70% of well and moderately differentiated squamous cell carcinomas as well as in 30% of the bronchioalveolar carcinomas.

3.3.7 Density of S100+ Langerhans dendritic cells in lymph nodes regional to lung tumours

Based on the histological evidence of immunological activity (Kitaichi et al., 1981), lymph nodes regional to lung tumours examined in this study were divided into three groups. Firstly lymph nodes with lymphocytic predominance, secondly lymph nodes with active germinal centre and thirdly unstimulated lymph nodes. S100+ LDC were found in the paracortical T lymphocyte area (Fig. 3.8). The density of S100+ LDC in various groups of lymph nodes are shown in Table 3.2.

The density of S100+ LDC was high in lymph nodes showing lymphocytic predominance and in lymph nodes with active germinal centres. The unstimulated lymph nodes showed the lowest density of these LDC. The density of the S100+ LDC in bronchial lymph nodes regional to normal lung was higher than that of the unstimulated lymph nodes.

The dendritic nature of LDC recorded in all varieties of lymph nodes was examined. 93% of LDC in lymph nodes with lymphocytic predominance (n= 25/27) showed more than three dendrites, compared to 89% in lymph nodes with active germinal centres (n= 41/46) and to less than 50% in unstimulated lymph nodes (n= 16/33). The number of dendrites reflected the state of immunological activity of these lymph nodes.

3.3.8 S100$^+$ Langerhans dendritic cells and patients survival with lung cancer

When patients were divided into those with the highest number of S100$^+$ LDC and a second group with the lowest number of LDC (Table 3.3), a number of comparisons was noted. The density of TIL was marked in tumours with the highest number of LDC and was scanty in tumours with the lowest number of LDC. The overall survival of patients in the former group was almost 8 fold that of the second group. 92.6% of the cases in the better surviving group were bronchioalveolar, well and moderately differentiated squamous cell carcinomas, while small cell lung cancer, poorly differentiated squamous cell and other varieties of adenocarcinomas formed all cases in the second group.

The male:female ratio in the two groups was 3:1, patients in both groups smoked an average of 28 cigarettes a day for an average of 37 years. Neither the number of cigarettes a day nor the number of years the patients smoked was related to the tumour type nor to the density of LDC in these tumours. There was no difference in tumour size or in the incidence of lymph node metastases in both groups.

3.4 Discussion

This study demonstrates that in lung tumours the density of antigen presenting S100$^+$ dendritic cells varies according to cellular subtype and differentiation of lung carcinomas. Bronchioalveolar, well and moderately differentiated squamous cell carcinomas contain the highest number of dendritic cells. The density of TIL is in direct proportional relationship with the number of S100$^+$ dendritic cells. Patients with high number of dendritic cells in their lung tumours e.g. bronchioalveolar, well and moderately differentiated squamous cell carcinomas survived longer than patients with a low number of dendritic cells e.g. poorly differentiated squamous and small cell lung cancer, i.e. 68.5 ± 32.9 months compared to 8.3 ± 7.7 months.

S100$^+$ LDC with more than 3 dendrites were the majority in bronchioalveolar, well, and moderately differentiated squamous cell carcinomas of the lung and in lymph nodes with lymphocytic predominance and active germinal centres. These findings favour the hypothesis that the increase in number of LDC dendrites in certain tumours may reflect an active immunological state, as it was observed, the increase in the number of fully dendritic LDC in lung bronchioalveolar, well and moderately differentiated squamous cell carcinomas was associated with marked TIL and tumour necrosis and this enhanced patients survival.

Contact between S100$^+$ LDC, tumours cells and T-lymphocytes was investigated in the varieties of lung tumours and in lymph nodes. Direct

contact between these antigen presenting cells and tumour cells was observed in all bronchioalveolar, well differentiated, moderately differentiated carcinomas and in the majority of adenocarcinomas. Direct contact was also observed between T-lymphocytes and LDC in lung tumours and in the T-zone of lymph nodes. This contact may reflect the immune interaction between LDC, tumour cells and T-lymphocytes. This has also been reported in human skin tumours (Gatter et al., 1983).

Central tumour necrosis in the investigated lung neoplasms was related to high density of $S100^+$ LDC and marked infiltration of TIL. This was observed in well, moderately differentiated squamous cell and in bronchioalveolar carcinomas. This may result from the effect of sensitized T-lymphocytes acting specifically against the target tumour cells (Stingl et al., 1987; Hart and McKenzie, 1990).

The current study shows the density of dendritic cells in lymph nodes regional to lung tumours is directly related to the immunohistological activity of these lymph nodes. Lymph nodes showing active germinal centres or lymphocytic predominance contained higher numbers of $S100^+$ dendritic cells than the unstimulated nodes.

Kitaichi et al., (1981) reported that the 3 years survival of patients in stage 2 and 3 of lung cancer in which the regional lymph nodes showed lymphocytic predominance or active germinal centres was better than patients with unstimulated lymph nodes.

Furukawa et al., (1981; 1984) also reported that patients with a marked number of S100+ LDC in and around their lung adenocarcinomas survived longer than patients without or with minimal infiltration of these cells. Temeck, et al., (1984) found 65.3% of patients with carcinomas of the lung alive for 10 years after diagnosis and treatment had epidermoid carcinomas. Patients with adenocarcinoma represented 33%, while there was only one patient with small cell lung cancer and one patient with large cell lung cancer alive. A similar study by Kern et al., (1986) reported 46.4% of patients with carcinomas of the lung who survived an average of 15.5 years after diagnosis and treatment had epidermoid carcinomas, 25.5% adenocarcinomas and 11.8% bronchioalveolar carcinomas. In a recent study of 9090 patients with carcinomas of the lung, Watkin et al. (1981) reported a longer survival for squamous cell carcinomas than small cell lung cancer. A relationship between the increased density of dendritic cells in other human tumours and longer patient survival has been reported during the last thirteen years e.g. nasopharyngeal tumours (Nomori et al., 1986), gastric tumours (Tsujitani et al., 1987), papillary thyroid carcinomas (Schroder et al., 1988), colorectal adenocarcinomas (Ambe et al., 1989), squamous cell carcinomas of the esophagus (Matsuda, 1990), cervical carcinoma (Xie, 1990), prostatic carcinoma (Bigotti et al., 1991), laryngeal carcinoma (Rucci et al., 1991) and transitional cell carcinoma of the urinary bladder (Inoue et al., 1994).

This study also demonstrated S100+ Langerhans dendritic cells are present in normal trachea, bronchus, normal lung parenchyma and normal bronchial lymph nodes. These dendritic cells were more commonly observed in the alveolar septum of normal lung but

occasionally were seen in the normal tracheal or bronchial mucosa. These observations are in agreement with Sertl et al., (1986), Richard et al., (1987) and Holt and Schon-Hegrad, (1987).

The primary function of Langerhans dendritic cells is the presentation of antigen to T-lymphocytes in the regional lymph nodes (Silberberg et al., 1976; Stingl et al., 1987). The selective increase in the number of LDC in bronchioalveolar, well and moderately differentiated squamous cell carcinomas may be related to specific tumour associated antigen (Sertl et al., 1986; Hart and McKenzie, 1990). Alternatively tumours may release factors which are chemotactic for dendritic cells, hence drawing them into the tumour environment (Halliday et al., 1992).

In conclusion this study has clearly demonstrated that the density of $S100^+$ LDC in lung tumours was related to cellular subtype and differentiation of tumour cells. Bronchioalveolar, well and moderately differentiated squamous cell carcinomas attracted the highest number of LDC, and this was associated with marked density of TIL and better survival. The mechanism by which the increased number of antigen presenting cells in tumours warrants detailed investigation.

3.5 SUMMARY

Antigen presenting $S100^+$ Langerhans dendritic cells were quantified in normal trachea, lung, bronchial lymph nodes, 130 lung tumours and in 100 lymph nodes regional to tumour. Dendritic cells were rarely seen as intraepithelial components of the normal bronchial mucosa, but more

commonly observed in the perivascular lymphoid tissue of the submucosa and in the alveolar septum of normal lung parenchyma (6 ± 4.85 cells/HPF). The density of these dendritic cells was marked in histologically normal bronchial lymph nodes. Bronchioalveolar, well and moderately differentiated squamous cell carcinomas contained the highest density of $S100^+$ dendritic cells, while small cell lung cancer and poorly differentiated squamous cell carcinoma showed the lowest density. Regional lymph nodes to lung tumours with lymphocytic predominance and active germinal centres showed the highest density of dendritic cells, while unstimulated lymph nodes contained the lowest number of $S100^+$ dendritic cells. Tumour infiltrating lymphocytes were marked in and around lung tumours with the higher density of dendritic cells. Survival of patients whose tumours contain high density of $S100^+$ dendritic cells was more favourable compared to tumours with low density of these cells. It is concluded that the density of the antigen presenting $S100^+$ dendritic cells in lung tumours was related to subtype and tumour differentiation. High dendritic cell densities was associated with longer patient survival.

CHAPTER 4

TOBACCO SMOKE INHALATION, LANGERHANS DENDRITIC CELLS, AND LUNG LESIONS

4.1 INTRODUCTION

Histopathological changes in the lungs induced by cigarette smoking have been documented in humans (The Health Consequences of Smoking, Cancer, Report of the Surgeon General, 1982, U.S. Department of Health and Human Services; Auerbach et al., 1957) and experimental animals (Leuchtenberger et al., 1961; Wynder et al., 1961). Auerbach et al., (1957) reported the tobacco smoke induced histologic changes in human trachiobronchial epithelium. These included basal cell hyperplasia, stratification and squamous cell metaplasia of the bronchial epithelium. The epithelial changes were more frequent in patients with lung cancer than in cancer free smokers.

The carcinogenic effects of the tobacco smoke on the lungs of laboratory animals have been investigated by many workers (Wynder, 1961 and Leuchtenberger, 1961). These studies have concentrated on tobacco smoke induced precancerous changes and tumour development. In mice, the tobacco smoke induced pulmonary changes described include bronchitis, atypical bronchial epithelial proliferation and occasional adenomatous lung tumours (Leuchtenberger, 1961).

In humans, Basset and Turiaf, (1965) together with Nezelof et al., (1973) identified the ultrastructure of cells consistent with that of LDC isolated from bronchioalveolar lavage fluid of patients with pulmonary histiocytosis X.

Casolaro, (1988), reported an increase in the number of $CD1^+$ dendritic cells in the bronchioalveolar lavage fluid of smokers, while Soler et al., (1989), described an increase in both $T6^+$ LDC and $M241^+$ dendritic cells in smokers lung.

A relationship between tobacco smoke inhalation and pulmonary interstitial granulomas has been reported in humans- 90% of patients with pulmonary histiocytosis X were smokers (Hance et al., 1988). An excellent review of LDC granulomatosis (histiocytosis X) was published by Hance et al., (1988). The three clinical syndromes, Letterer-Siwe disease, Hand-Schuller-Christian syndrome and eosinophilic granuloma were grouped and called histiocytosis X, based on the similarities of the lesions (Faber, 1941; Lichtenstein, 1953). Pulmonary Langerhans cell granulomatosis (pulmonary histiocytosis X) is slightly different from the other varieties of histiocytosis X. The former is commonly seen in young male adults (Smith et al., 1974), but not in children as are the other forms of histiocytosis X.

Katzenstein and Askin, (1990) described detailed histologic features of pulmonary eosinophilic granuloma which included bronchiocentric, patchy and nodular lesions due to interstitial infiltration with a mixture of histiocyte-like cells and variable number of eosinophils, plasma cells and lymphocytes. The nodular interstitial lesions may appear star-shaped due to infiltration and fibrosis along the alveolar septum. A DIP-like reaction (desquamative interstitial pneumonia), with accumulation of alveolar macrophages in the surrounding alveolar spaces may be seen but is not specific for pulmonary LDC granulomatosis. Bronchiolitis is frequently observed and spontaneous healing of pulmonary LDC

granulomatosis may take place leaving a starfish-shaped scar, (Katzenstein and Askin, 1990).

The diagnostic cell in this lesion is the LDC or histiocytosis-X cell. It is large cell with an ill-defined border, abundant eosinophilic cytoplasm, and a folded or indented nucleus with an inconspicuous nucleolus. There are many of these cells in active lesions, but become rare in older lesions with fibrosis (Katzenstein and Askin, 1990). Birbeck granules in both epidermal LDC and histiocytosis X cells show the same affinity for zinc-iodide-osmium (ZIO) staining (Wolf, 1972), both cells are S100 protein positive (Flint et al., 1986, 1987) and OKT6 positive (Soler et al., 1989). The ultrastructural features of histiocytosis X cell are consistent with that of LDC (Wolf K., 1972 and Flint, et al., 1986 & 1987). However the BG in the histiocytosis X cell appear to be more often attached to the cell membrane than those in epidermal LDC.

The aim of this study was to investigate experimentally the effect of tobacco smoke on pulmonary LDC in mice. The associated pulmonary lesions which developed are also described.

4.2 MATERIALS AND METHODS

4.2.1 Animals

BALB/c female mice 6-8 weeks old were obtained from the central Animal House at the University of Tasmania. The 108 mice used for the tobacco smoke inhalation experiments were divided into two groups, experimental and controls, each of 54 mice. Control mice were kept in groups of 12, while the experimental mice were kept in groups of 6 Mice.

4.2.2 Experimental design

The details of this experiment are outlined in Section 2.7

4.2.3 Procedure to visualise Langerhans dendritic cells

A modified ZIO procedure of Champy, 1913; Rodriguez and Caorsi, 1978 was developed because of the nature the lung. The details of this procedure are listed in Chapter 2. The number of ZIO^+ LDC in four high power fields (HPF X400) was calculated. The mean and the standard deviation were statistically analysed for groups of mice assessed.

4.3 Results

4.3.1 Tobacco smoke induced pulmonary changes

Bronchial epithelial hyperplasia, focal alveolar cell hyperplasia, mononuclear and eosinophil infiltration of the alveolar septum, together with alveolar macrophages in the alveolar spaces were increasingly observed in lungs of mice after 4 weeks of exposure to tobacco smoke. By the end of week 8, multifocal interstitial lung lesions were present composed of monocytes and lymphocytes (Fig. 4.1). This was observed in the lungs of all six mice examined.

After 12 weeks of tobacco smoke exposure, interstitial granulomas without central necrosis or cavitation were observed in lungs of 3 of 6 mice. Cholesterol-like crystals (Fig. 4.2) were present between the cells forming these granulomas; histiocyte-like cells, foamy macrophages, lymphocytes, plasma cells and eosinophils. The alveolar spaces around

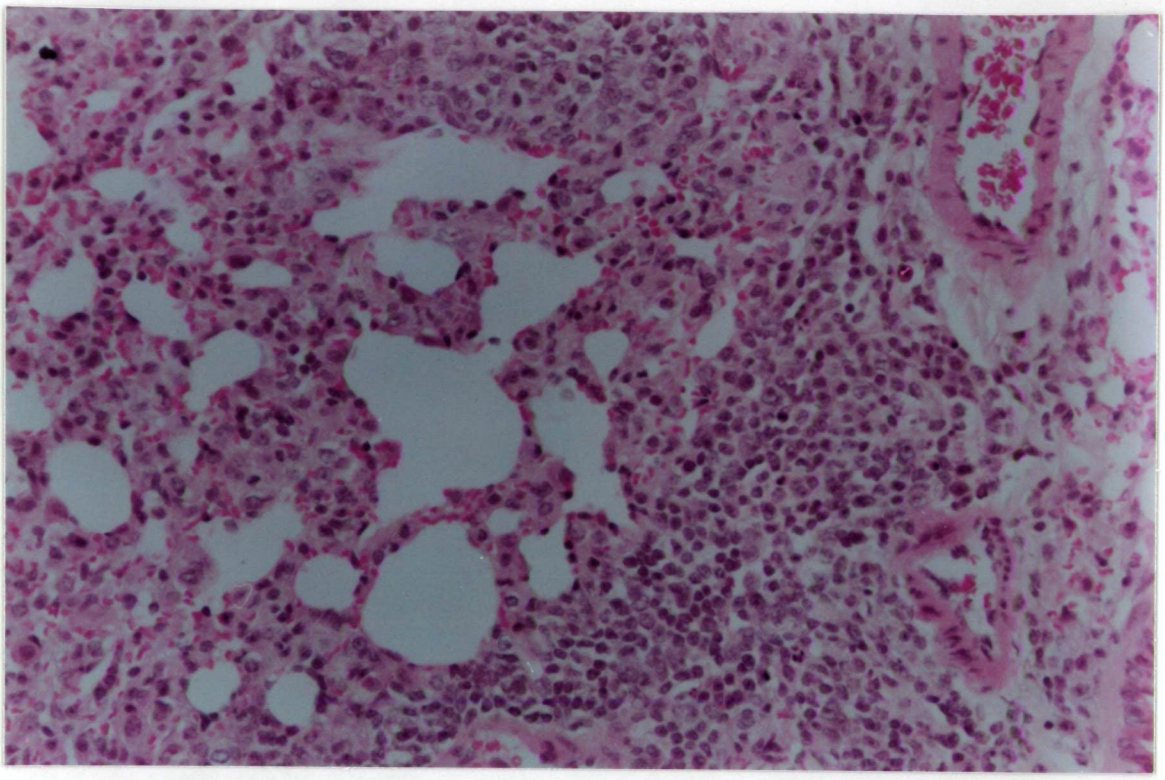


Figure 4.1 An increase in thickness of the alveolar septum due to increased numbers of mononuclear cells. (H&E X 100)

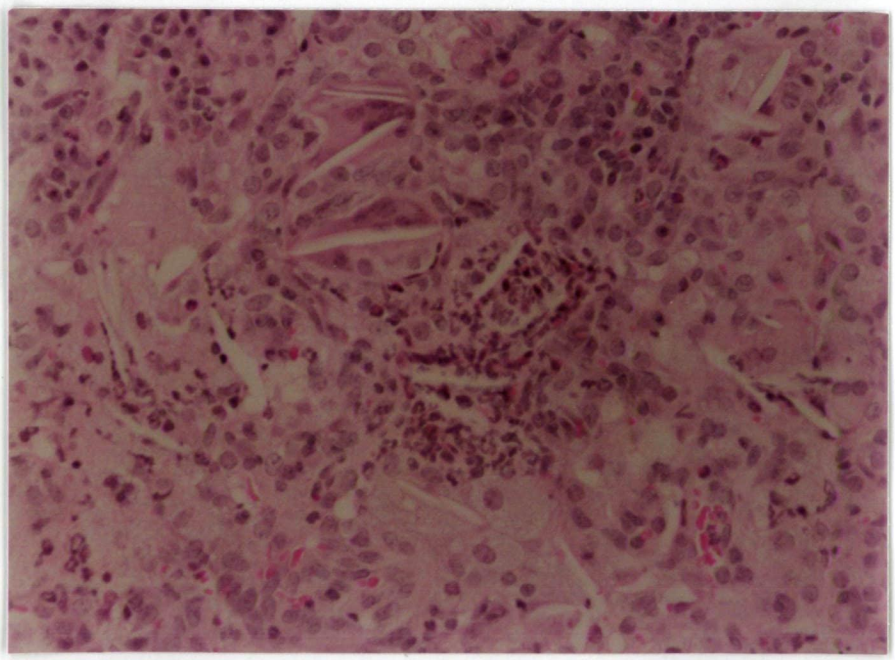


Figure 4.2 Interstitial granuloma showing cholesterol crystals, mononuclear cells and foamy alveolar macrophages. (H&E X 200)

the granulomas showed alveolar epithelial hyperplasia with marked accumulation of macrophages.

By 16 weeks, bronchial epithelial hyperplasia and occasional focal bronchial squamous metaplasia were observed (5 of 6 mice). The lung of one mouse at this stage showed a complex of histopathological changes. The basic lesion was peribronchial chronic inflammation (Fig. 4.3) some with granulomas and a central cavity (Fig. 4.4). The cavity resulted from deformed bronchioles by the peribronchial inflammatory infiltration as some ciliated columnar epithelium was still identifiable. The cellular infiltrate of the granuloma extended in the adjacent alveolar septum forming a star-shaped lesion (Fig. 4.5). The cells forming the granuloma included clusters of large histiocyte-like cells and macrophages containing PAS positive material and haemosiderin. The histiocyte-like cells (Fig. 4.6) were large with an irregular and indented nucleus with occasional prominent nucleoli. The cytoplasm appeared pale acidophilic with ill-defined short dendrites extending from the cell membrane.

Some bronchioles adjacent to the inflammatory lesions were filled with acute inflammatory exudate (Fig. 4.4) and the alveolar spaces contained a marked number of alveolar macrophages (DIP-like reaction, Fig. 4.7). The alveolar epithelium was hyperplastic and the subpleural airspaces showed focal emphysematous changes.

By the end of 20 weeks, 2 of 6 mice showed marked bronchial epithelial hyperplasia together with squamous metaplasia with occasional atypical epithelium. Multifocal interstitial granulomatous lesions were observed in the lungs of 2 of 6 mice similar to that found after 16 weeks. Older interstitial lesions demonstrated typical satellite or star-shaped fibrosis.

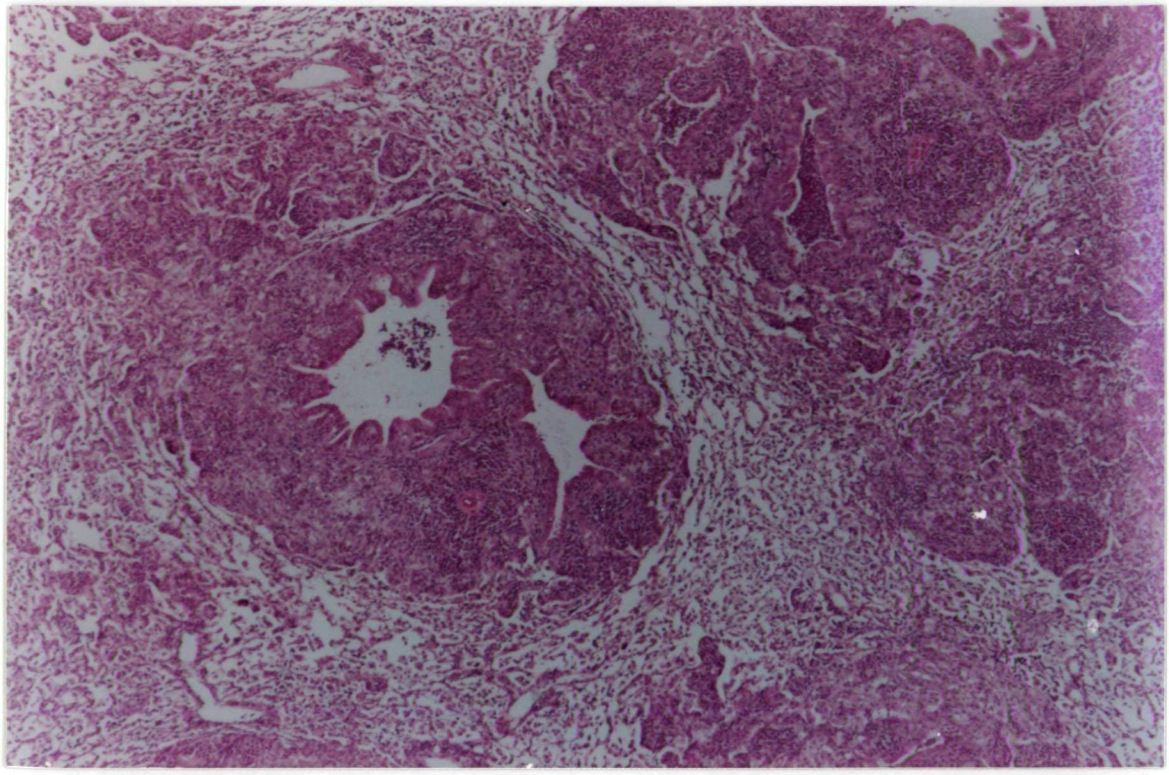


Figure 4.3 Multiple peribronchial interstitial granulomatous inflammation. (H&E X 100)

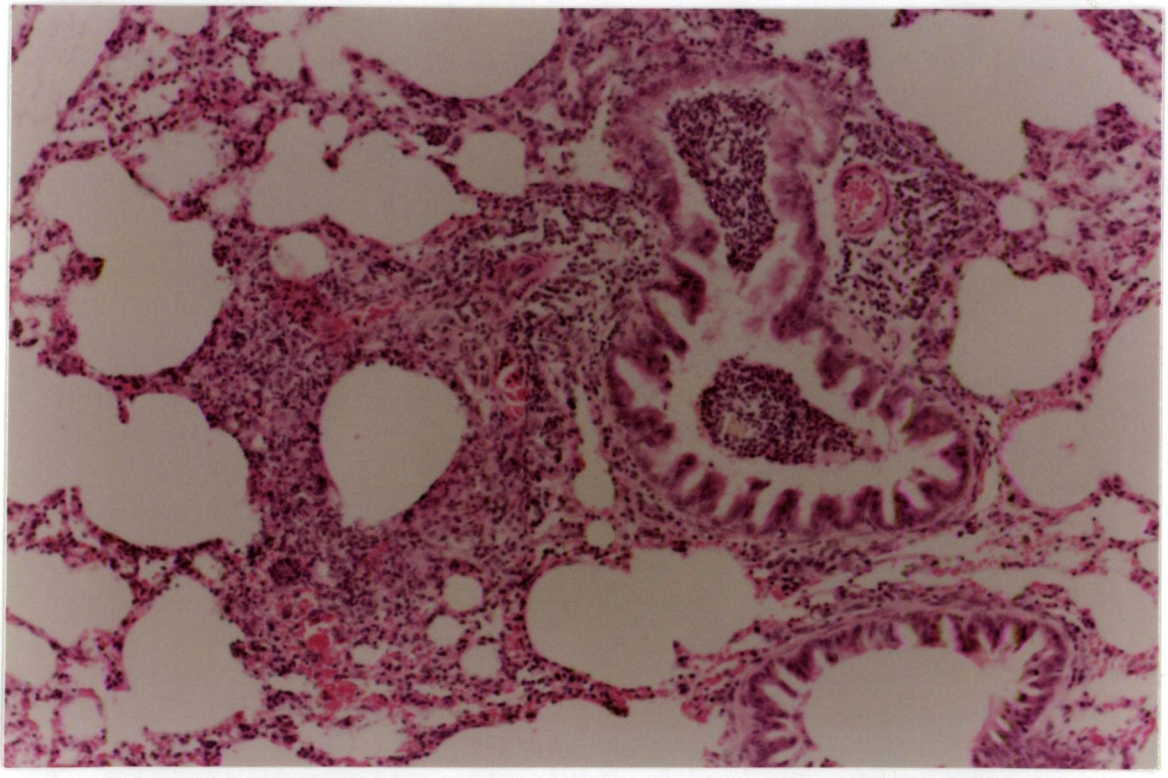


Figure 4.4 Interstitial pulmonary granuloma with a central cavity. Polymorphs present in bronchial lumen. (H&E X 200)

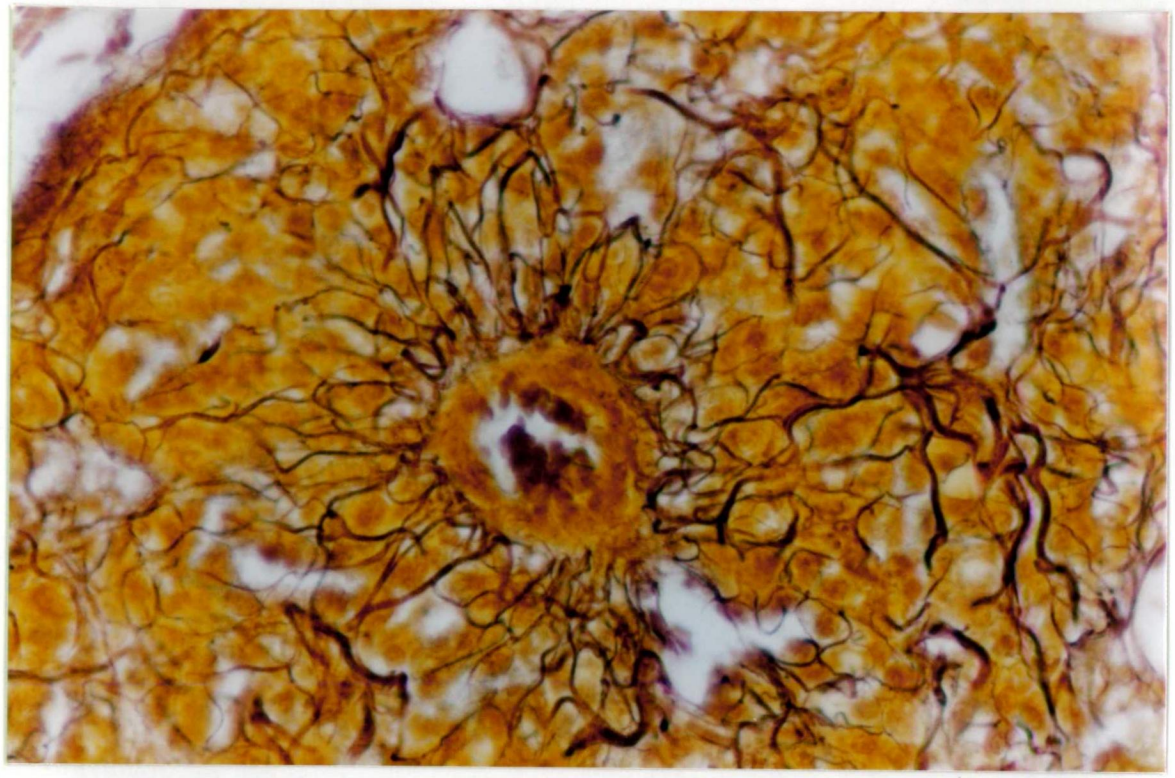


Figure 4.5 Starfish-like lesion of the interstitial pulmonary granuloma. (Gomori's reticulum stain X 400)

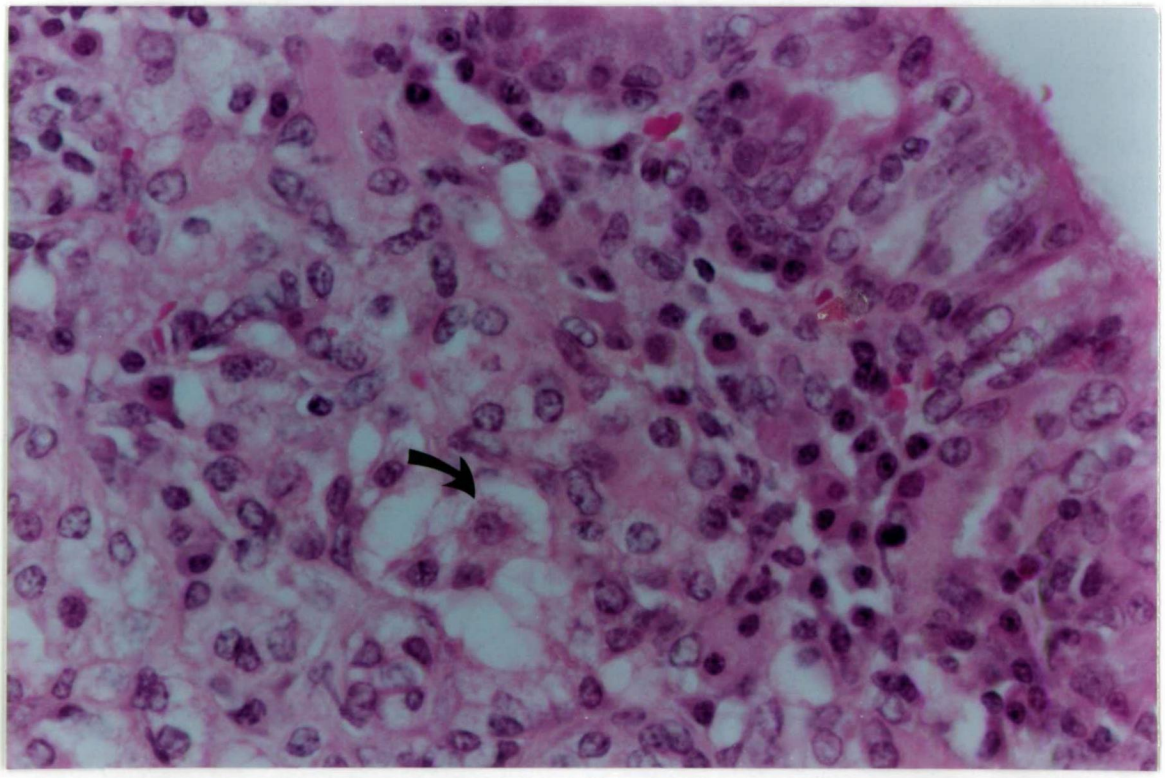


Figure 4.6 Langerhans dendritic cells (arrow) in peribronchial interstitial granuloma. (H&E X 400)

Neoplastic-like lesions were found in the lungs of two mice. One lesion consisted of proliferating alveolar epithelial cells bounded by chronic inflammation (Fig. 4.8). These histopathologic features are similar to so called alveogenic lung tumours (Shimkin and Stoner, 1975), although the nature of these lesions are in-question i.e. reactive or neoplastic.

The lesion in the lung of the second mouse was a single microscopic lesion consisting of multiple papillary structures (Fig. 4.9), filling what appeared to be a bronchiolar lumen. The cells of this lesion were cuboidal to columnar in shape and the nuclei hyperchromatic, rounded and eccentrically located. There were no cilia extending from the free borders of these cells. The histologic features of this lesion were consistent with a Clara cell bronchial adenoma.

At week 20, tobacco smoke exposure ceased and the remaining mice (6) were kept for a further 6 weeks. The alveolar epithelial hyperplasia, the interstitial cellular accumulation, interstitial granulomas, features of bronchiolitis, DIP and the emphysematous changes resolved in 75% of the mice (5/6). A mild degree of these changes was observed in the remaining mouse. Only bronchial epithelial metaplasia persisted.

4.3.2 Pulmonary ZIO$^+$ Langerhans dendritic cells

The ZIO$^+$ dendritic cells were smaller than alveolar macrophages and most were oval or triangular in shape. Black granules (Fig. 4.10) were observed scattered in the cytoplasm. The dendritic nature of these cells was demonstrated on serial sectioning. The nuclear:cytoplasmic ratio was always low around 1:2; the nucleus appeared irregular. Most of the ZIO$^+$ LDC were found in the alveolar septum, infrequently in alveolar spaces

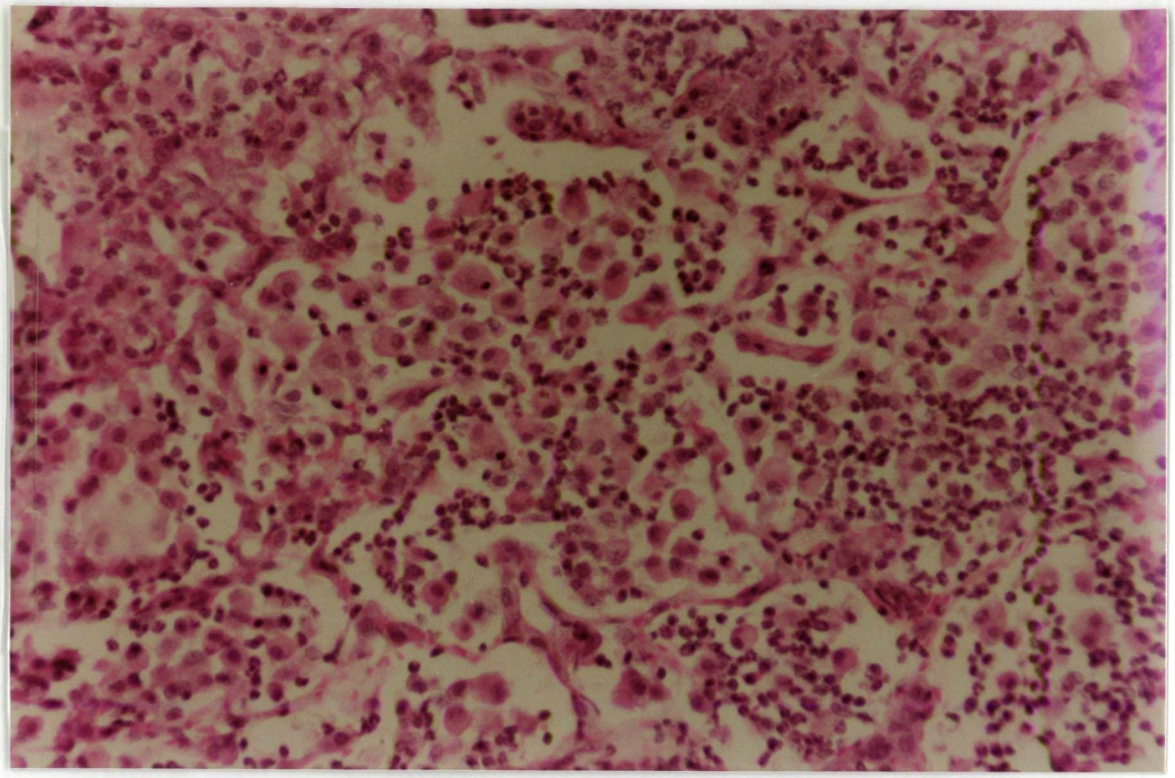


Figure 4.7 Desquamative interstitial pneumonia (DIP) in the lung parenchyma. (H&E X 200)

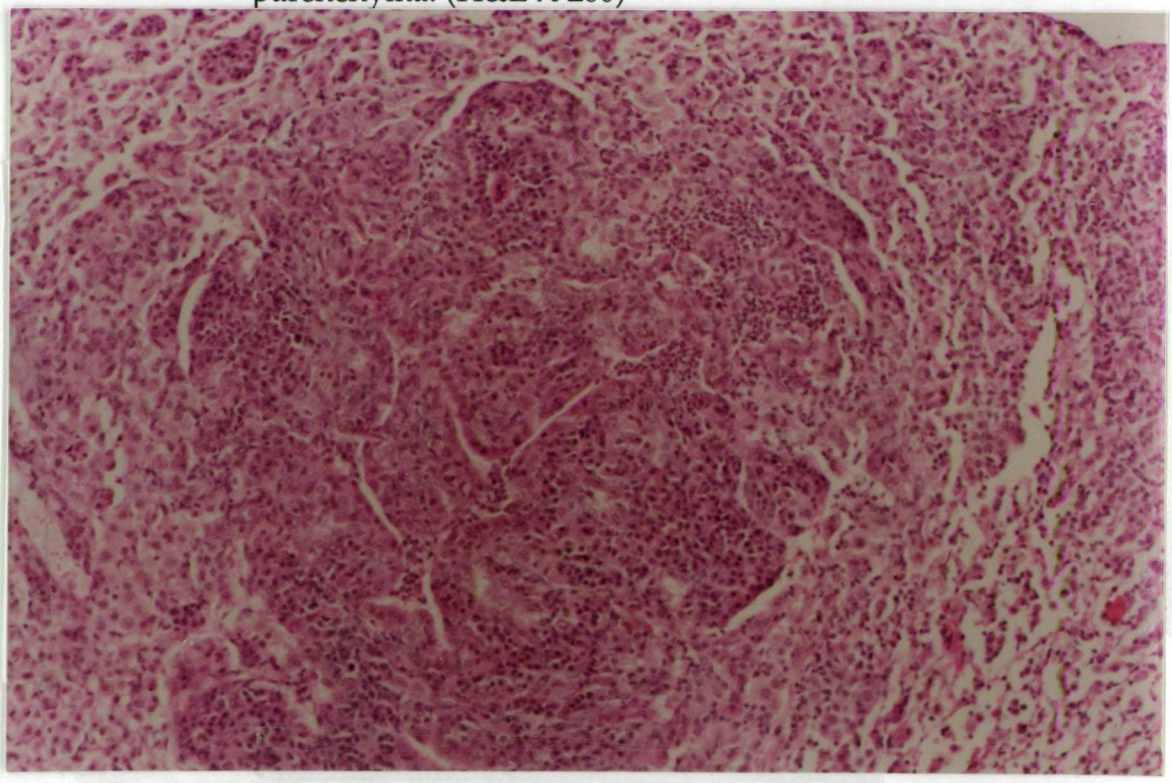


Figure 4.8 Alveolar proliferative cells bounded by inflammatory reaction. (H&E X 100)

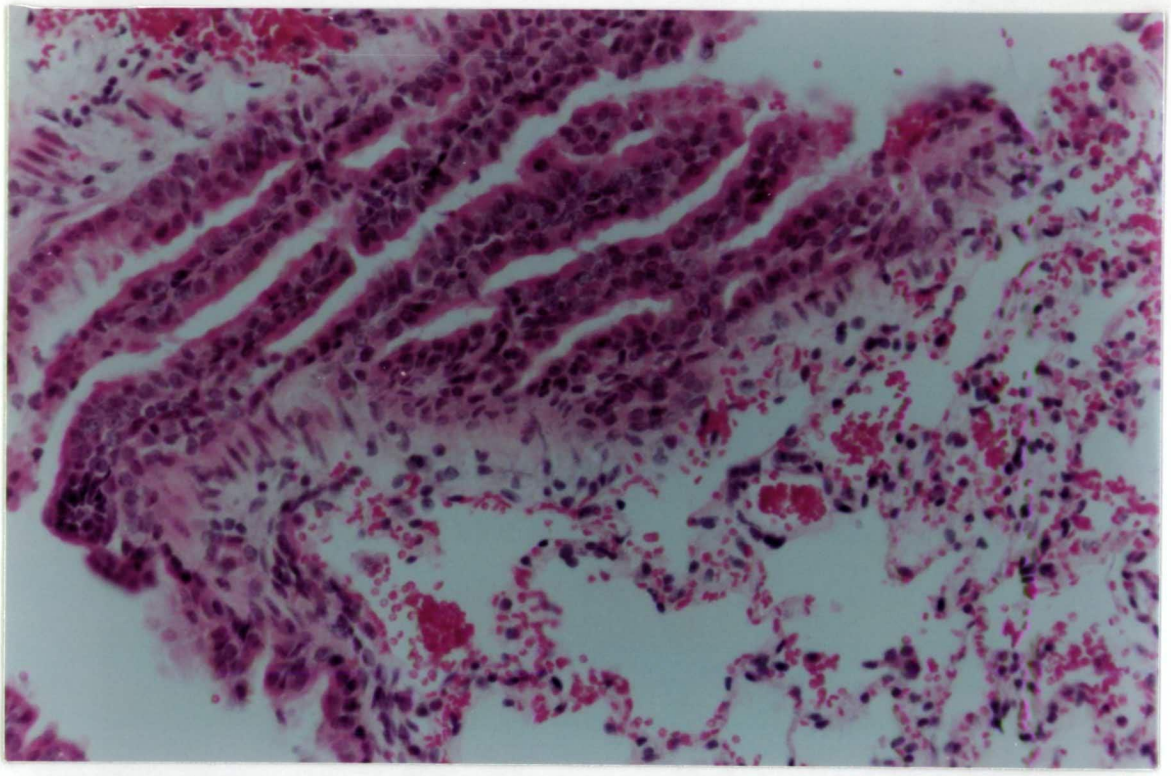


Figure 4.9 Papillary Clara cell adenoma. (H&E X 400)

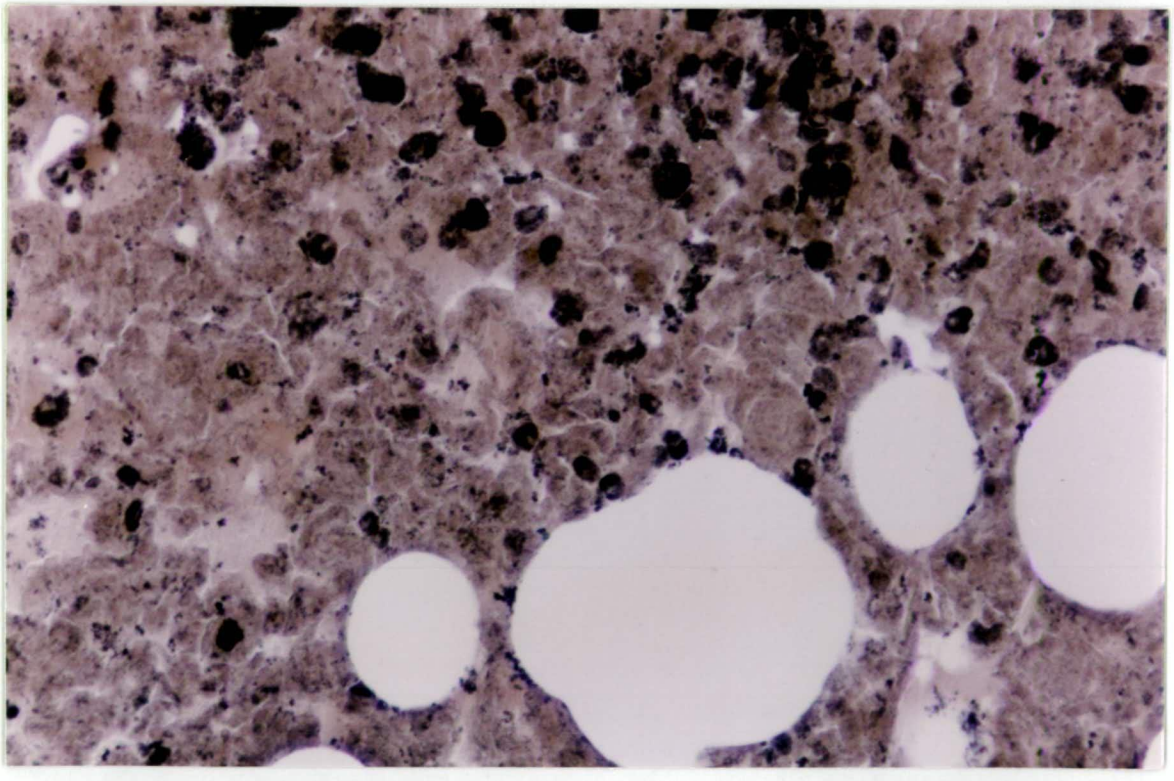
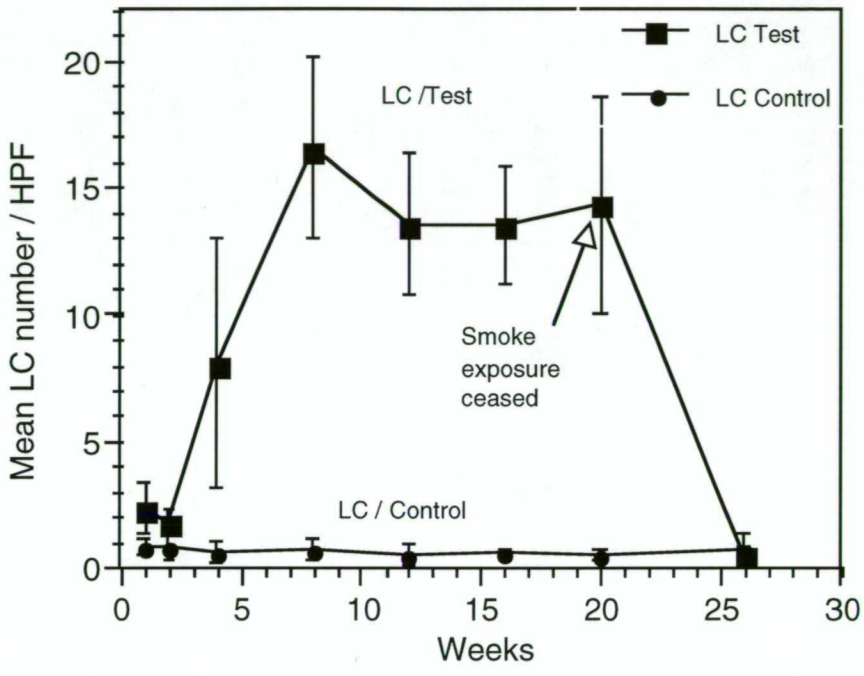


Figure 4.10 ZIO positive dendritic cells in lung granulomatous inflammation after exposure to tobacco smoke. (ZIO staining X 200)

Figure 4.11 Pulmonary murine LDC after exposure to tobacco smoke



Analysis of variance between test and control group showed a statistically significant difference in LDC number ($p<0.0001$) between 4-20 weeks

and rarely in the bronchial epithelium. The mean number of ZIO^+ LDC in normal lung was 1.3 LDC ± 0.4/HPF.

There was a steady increase in the number of ZIO^+ LDC mainly in the thickened alveolar septum of the tobacco smoke exposed mice (Fig. 4.11) starting from the end of the first week (test = 2.3 ± 1.0; control = 0.8± 0.3). After eight weeks of tobacco smoke exposure, the number of pulmonary LDC peaked (test = 16.5 ± 3.6 ; control = 0.7 ± 0.4, $p < 0.0001$) and remained high with continuos exposure to tobacco smoke (test = 14.3 ± 4.3; control = 0.5 ± 0.2, $p < 0.0001$). When exposure to tobacco smoke ceased at the end of the 20 weeks, the number of ZIO^+ LDC in the lungs of the remaining mice decreased to the level of the control group, (test = 0.5 ± 0.3; control = 0.7 ± 0.6) after a further six weeks.

On ultrastructure, the pulmonary dendritic cells with positive ZIO staining showed all the ultrastructural features of LDC; the indented large irregular nucleus and black BG were easily identifiable (Figs. 4.12 and 4.13). Some branched BG were observed near the nuclear membrane. Many tubular structures were identified in the cytoplasm and the mitochondria were numerous.

4.3.3 Ia positive pulmonary LDC

The morphology and size of the Ia^+ dendritic cells were consistent with that of ZIO^+ LDC. Ia^+ cells were mainly found in the alveolar septum and were occasionally seen with a few dendrites. Their numbers were similar to that of the ZIO^+ LDC.

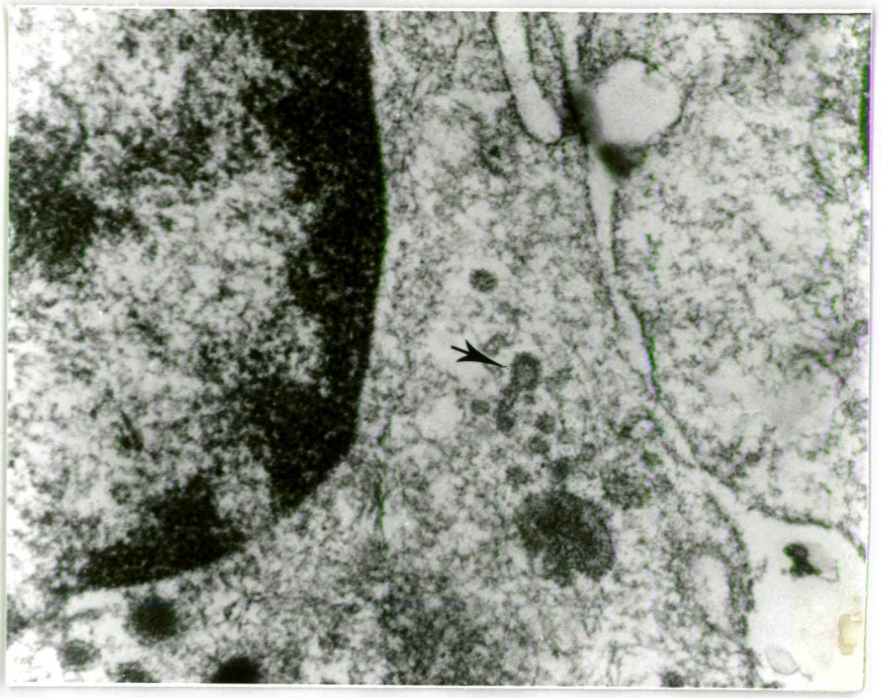


Figure 4.12 ZIO positive pulmonary dendritic cell showing labelled (arrow) Birbeck's granules. (V-7.4-ZIO staining X 34,000)

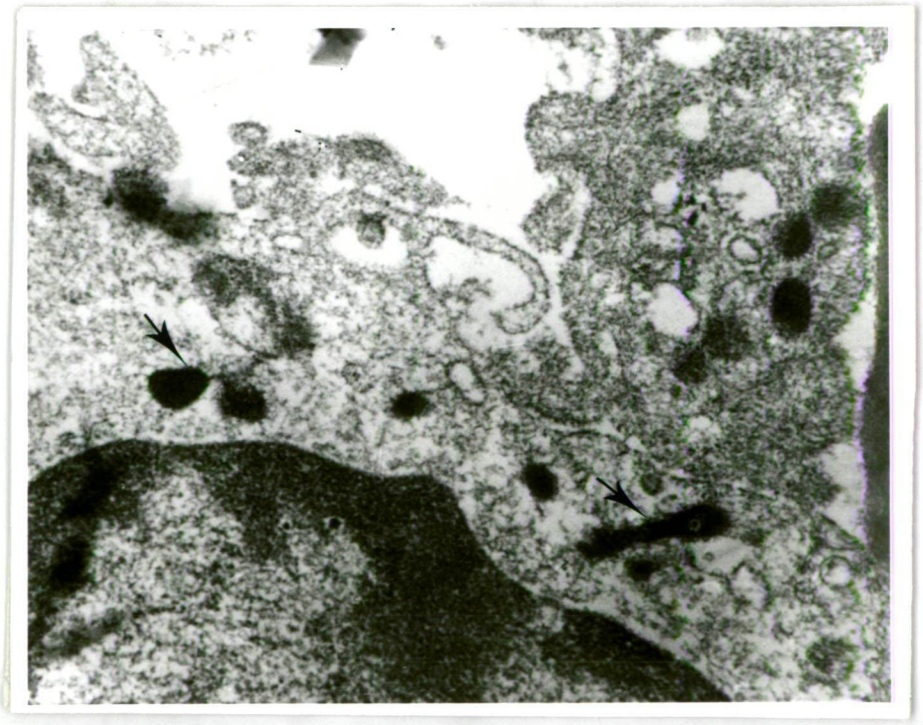


Figure 4.13 ZIO positive pulmonary dendritic cell showing branched and unbranched labelled (arrow) Birbeck's granules. (V-7.4-ZIO staining X 34,000)

4.4 Discussion

The number of dendritic LDC in the lungs of mice exposed to tobacco smoke for twenty weeks increased approximately 25 fold. Although the increase in the pulmonary LDC was observed within one week, the highest level was obtained after 8 weeks and remained elevated until smoke inhalation treatment ceased at 20 weeks. This is in agreement with Casolaro et al., (1988), and Soler, (1989), who reported an increase in LDC in smokers lungs in human.

In this study ZIO^+ pulmonary LDC were always observed in control lungs in the alveolar septum and rarely in the bronchial epithelium. The mean of ZIO^+ pulmonary LDC in normal murine lung was 0.9 ± 0.4 cells/HPF. This parallels the previous finding of low density of pulmonary LDC in normal human lung (Chapter 3).

The increase in the ZIO^+ pulmonary LDC after tobacco smoke exposure was associated with the development of interstitial granulomas and inflammation. The alveolar spaces around the granulomas showed alveolar epithelial hyperplasia with marked accumulation of alveolar macrophages. By 16 weeks, the basic lesion was the interstitial granuloma with a central cavity together with inflammatory reaction of the lung parenchyma around the granuloma. The cellular infiltrate of the granuloma extended into the adjacent alveolar septum forming star-shaped lesions.

The histiocyte-like cells on cytological features were consistent with those of the histiocytosis-X cell (Katzenstein and Askin, 1990). They were irregular, with an indented or lobulated nucleus, pale acidophilic

cytoplasm with many ill-defined short dendrites extending from the cell membrane. While there were many of these cells in active lesions, they become rare in older lesions with fibrosis.

In this study, on ultrastructural examination of the ZIO^+ pulmonary dendritic cells, BG were observed in the cytoplasm (Figs. 4.12 and 4.13). This confirmed the lineage relationship between histiocytosis X cell and LDC.

The bronchioles associated with the granulomas were filled with acute inflammatory exudate and the alveolar spaces contained a marked number of alveolar macrophages (DIP-like reaction). The alveolar epithelium was hyperplastic. By the end of 20 weeks, some older interstitial granulomas progressed into typical fibrotic satellite or star-shaped lesions. These histopathological features are consistent with the tobacco smoke related human clinical syndrome, pulmonary histiocytosis X (Hance et al., 1988 & Katzenstein and Askin, 1990), as called pulmonary LDC granulomatosis (Lieberman, 1980).

Although the pathogensis of pulmonary LDC granulomatosis is not clear, 90% of patients are smokers (Hoffman and Hect, 1985; Hance et al., 1988). Carcinogens of the tobacco smoke e.g. benzo[a]pyrene and catechols may cause alveolar epithelial damage leading to proliferative, hyperplastic and neoplastic alveolar epithelial cells (Favara et al., 1983; Hoffman and Hect, 1985; Hance et al., 1988). The damaged alveolar epithelium may express neoantigens which triggered the LDC response and T- lymphocyte activation leading to necrosis of the target cells. Activated macrophages secreting collagenase, elastase, prostaglandins and other factors are also central to granuloma formation (Favara et al., 1983; Hance et al., 1988).

Benzo(a)pyrene (BP) while increasing the number of pulmonary LDC, may alter cellular immunity via prostaglandins (Ruby et al., 1989; Andrews et al., 1991). Ruby et al., (1989), and Andrews et al., (1991), reported BP and co-carcinogen catechols increased the number of Ia^+ LDC, changed their morphology and impaired their function. This was associated with the development of skin tumours after 24 weeks of treatment. Andrews and colleagues (1991), implanted the prostaglandin synthetase inhibitor, indomethacin beneath mouse skin before treatment with BP. This restored contact hypersensitivity; delayed the onset and reduced the size of skin tumours induced by BP. Tobacco smoke condensate (TSC) induced cutaneous carcinogenesis and changes in epidermal LDC. TSC increased the LDC density, altered their morphology and impaired their function. This was associated with skin tumour development (Chapter 5).

Studies on the carcinogenic effects of tobacco smoke on the lungs of laboratory animals have concentrated on precancerous changes and tumour development (Wynder, 1961; Leuchtenberger, 1961; Mostofi and Larsen, 1951). Interstitial granulomas have been observed only in one previous study by Mostofi and Larsen (1951). They were investigating the histopathogenesis of urethane induced pulmonary tumours in mice where they observed the formation of interstitial granulomas between 3-12 weeks prior to tumour development. They also observed large acidophilic cells in these granulomas. While Mostofi and Larsen, (1951), did not investigate their observation further, their description of the interstitial granuloma is consistent with that of pulmonary LDC granulomatosis (histiocytosis X). The large acidophilic cells are atypical LDC (Lieberman, 1980 and Shimkin and Stoner, 1975).

In this study, the development of tumour-like lesions followed the formation of the interstitial granuloma after 20 weeks exposure to the tobacco smoke was observed. The histopathologic features of these lesions were consistent with bronchial Clara cell adenoma and so called alveogenic tumours. This was associated with a high density of ZIO^+ pulmonary LDC. Shimkin and Stoner (1975), both reported 95% of lung tumours in mice originate from the alveolar epithelium. High density of $S100^+$ LDC in tobacco smoke related human bronchioalveolar carcinoma and well differentiated squamous cell carcinoma was described in Chapter 3. Consequently, a strong relationship between tumour type and the density of tumour infiltrating LDC is confirmed.

While tobacco smoke increased the ZIO^+ pulmonary LDC density, the impaired function of these cells induced by benzo(a)pyrene in the inhaled tobacco smoke may have resulted in disordered alveolar epithelial growth in a setting of compromised immunological function. After tobacco smoke exposure ceased the histopathological changes resolved and the density of pulmonary LDC returned to that of control levels. Only the bronchial epithelial metaplasia did not show reversible change. Bronchial epithelial metaplasia may require a longer time to resolve.

In conclusion this study has demonstrated that tobacco smoke increased the density of ZIO^+ pulmonary dendritic cells and this was associated with the development of bronchial squamous metaplasia, interstitial granulomatous lesions (pulmonary LDC granulomatosis) and alveolar cell proliferation. The density of pulmonary LDC returned to that of the control level after ceasing tobacco smoke exposure and the interstitial granulomatous lesions, but not the metaplasia resolved. The mechanism

by which tobacco smoke increased the density of pulmonary LDC may be related to benzo(a)pyrene, while functional changes in these antigen presenting cells may have a role both in chronic granulomatous inflammation and tumour development.

4.5 Summary

The density of ZIO^+ pulmonary Langerhans dendritic cells was increased approximately twenty fold in mice after passive exposure to tobacco smoke. This was associated with pulmonary changes consistent with the cigarette smoking related clinical syndrome in humans, pulmonary Langerhans cell granulomatosis. The major feature was an interstitial peribronchial granuloma. The cellular infiltrate of the granuloma (lymphocytes, plasma cells, eosinophils, clusters of large histiocyte-like cells and macrophages) extended into the adjacent alveolar septum forming a star-shaped lesion. The histiocyte-like cells were large with pale acidophilic cytoplasm and many ill-defined short dendrites extending from the cell membrane. Bronchial epithelial metaplasia also developed. The interstitial changes were followed by the development of proliferative alveolar and bronchial lesions in two mice.

The ZIO^+ cells were consistent with Ia^+ pulmonary dendritic cells and their ultrastructure was similar to that of pulmonary Langerhans cells. After ceasing exposure to tobacco smoke the density of pulmonary Langerhans cells returned to that of the control level; interstitial granulomatous lesions disappeared, but the bronchial epithelial metaplasia did not reverse. Tobacco smoke exposure of mice produces interstitial granulomatous inflammation similar to Langerhans cell

granulomatosis in humans. The elevated level of pulmonary Langerhans cells implicate these cells in the pathogenesis of these lesions.

CHAPTER 5

TOBACCO SMOKE CONDENSATE, LANGERHANS DENDRITIC CELLS AND SKIN TUMOURS

5.1 Introduction

Benzo[a]pyrene and catechols are the most important of the 71 carcinogens in tobacco smoke condensate (TSC; Dube and Green, 1982). Skin papillomas and squamous cell carcinomas of the skin have been reported in various animals e.g. guinea pigs, Swiss mice, C57 black mice, after treatment with TSC (Wynder et al., 1953, 1961; Drukrey, 1961; Day, 1966).

Ruby et al., (1989) and Andrews et al., (1991) studied the effect of the tobacco derived benzo[a]pyrene (BP) and co-carcinogen catechols on the number, morphology and function of epidermal Langerhans cells (LDC). They reported a significant increase in number of the Ia^+ LDC, changes in their morphology and impairment of their function. This was associated with the development of skin tumours after 24 weeks of treatment (58% were squamous cell papilloma and 42% squamous cell carcinoma).

The various reports on the density of LDC in squamous cell carcinomas of human skin are conflicting. Gatter et al., (1984) used NA1/34 monoclonal antibody to identify LDC in human skin tumours and reported changes in LDC morphology and depletion in their number in squamous cell carcinomas. Smolle et al., (1986) used OKT-6 to study LDC in human skin tumours and reported a low density of LDC in squamous cell carcinomas compared to normal skin. McArdle and co-workers,

(1986) used an antibody to S100 protein to study LDC in various skin lesions and reported a high LDC density in keratoacanthoma, squamous cell carcinomas and basal cell carcinomas compared to the normal epidermis. Korenberg and his colleagues (1987) also used an antibody to S100 protein to study LDC and supported McArdle et al., (1986) in regard to the high density of LDC in keratoacanthoma but found a low density of $S100^+$ LDC in squamous cell carcinomas. Alcalay et al., (1989) used adenosine triphosphatase to study LDC and reported changes in LDC morphology but no increase in LDC number in squamous cell carcinomas.

Analysis of LDC in cervical intraepithelial neoplasia likewise are conflicting. The majority of reports indicate high LDC number in cervical intraepithelial neoplasia (Morris et al., 1983; Caorsi and Figueroa, 1984, 1986; McArdle and Muller, 1986; Bonilla-Musoles et al., 1987 and Xie, 1990), but Tay et al., (1987) reported low LDC numbers in these lesions. The variation in the number of LDC in squamous cell carcinoma in these reports may reflect the different methods used including counting techniques.

The present investigation arose out of the previous study (chapter 3) where an increase in LDC density in well and moderately differentiated human lung squamous cell carcinomas was demonstrated and this was associated with increased patient survival (Chapter 3). As tobacco smoke is linked to the cause of these tumours (Auerbach et al., 1957), the present investigation was designed to examine the effect of TSC on LDC during

carcinogenesis in murine skin, a well established model to analyse the sequential development of squamous tumours.

5.2 Materials and Methods

5.2.1 Animals

Two hundred and four BALB/c female mice 6-8 weeks old were obtained from the Central Animal House at the University of Tasmania after ethical approval. Forty eight mice were used to assess the function of LDC. To assess the effects of TSC on cutaneous LDC, 2 groups of 78 mice each, experimental and control were used (Section 2.16).

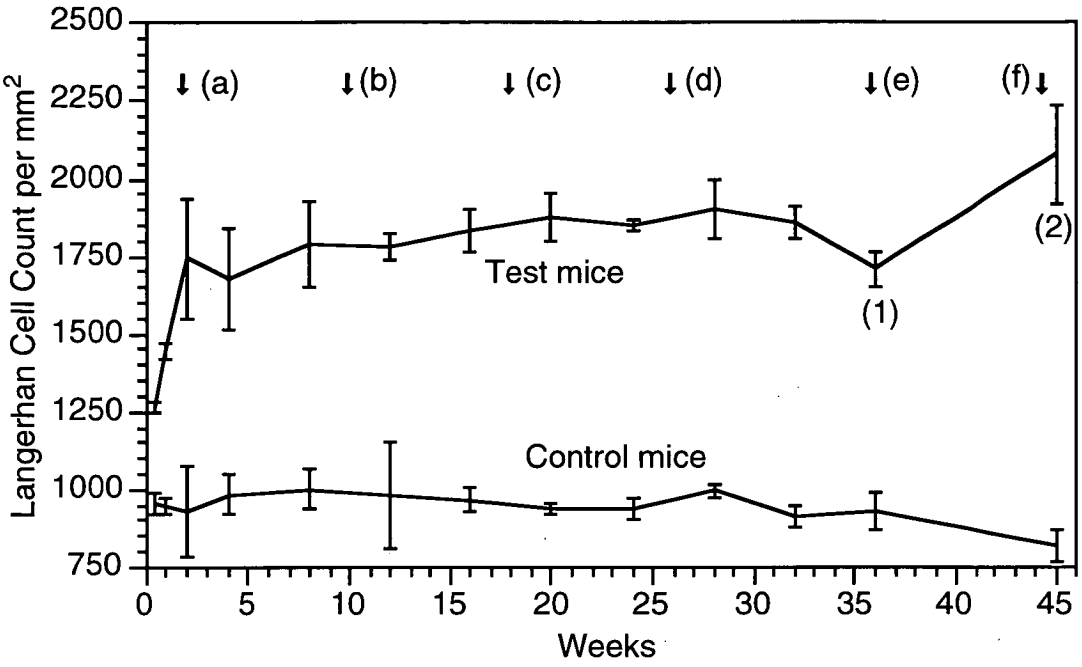
5.2.2 Experimental design

TSC was applied every second day including the weekends. At 35 weeks TSC treatment was stopped, and mice were kept for a further 10 weeks to observe the changes in tumour size and the relationship to LDC numbers (Details are given in Section 2.18).

5.2.3 Identification and enumeration of Ia positive LDC in epidermal sheets

The Ia^+ LDC were identified in the epidermal sheets as brown dendritic cells with various numbers of dendrites extending from the cell body.

Figure 5. 1 Skin lesions and changes in LDC number induced by TSC



(a) Epidermal hyperplasia.
(b) Dysplastic changes.
(c) Skin papilloma.
(d) Early invasive squamous cell carcinoma.
(e) Treatment with TSC stopped.
(f) Ten weeks after stopping TSC treatment.

Difference in LDC number in both test and control groups is significant ($p < 0.0001$).

The increase in LDC number in the test mice, ten weeks after stopping TSC treatment (2) is significant compared with LDC number at 36 weeks (1), $p < 0.0001$.

5.2.4 Contact sensitivity assessment

The function of LDC was assessed by inducing a contact hypersensitivity reaction at the 2nd and the 6th weeks, after treatment with TSC in a 1:1 solution of acetone:olive oil or in the control mice, acetone:olive oil alone. Details are listed in Section 2.24.

5.3 RESULTS

5.3.1 Skin Pathology induced by tobacco smoke condensate (TSC)

The histopathological changes induced by the TSC in the skin of the treated mice are summarised in Fig. 5.1. Epidermal hyperplasia was observed two weeks after treatment; the epidermis was composed of 6-8 cell layers compared to 2-3 layers of control epidermis.

Dysplastic changes in the hyperplastic epidermis were observed in 25% of mice after 10 weeks of TSC treatment. The cells had enlarged hyperchromatic nuclei, a high nuclear:cytoplasmic ratio and involved various thickness of the epidermis. By 18 weeks about one third of the treated mice developed macroscopic skin tumours: small wart-like papillomas, up to 2 mm in diameter.

After 26 weeks of treatment, the skin of all remaining mice showed skin tumours of various sizes, between 1-6 mm in diameter. The histology of the largest skin tumour showed early invasive squamous cell carcinoma. By 35 weeks, the size of the skin tumours of all treated mice was between

0.2-1.3 cm in diameter and one third of mice had developed multiple tumours (Fig. 5.2a). The histology of skin tumours of the six mice killed at this stage showed invasive squamous cell carcinoma (Fig. 5.3).

Application of TSC was stopped by 35 weeks and 48 mice were kept under observation for a further 10 weeks. Macroscopically, 23% of tumours spontaneously disappeared, the remaining tumours showed about 50% reduction in their size (Fig. 5.2b). Histopathologic examination of these lesions revealed invasive squamous cell carcinomas of the skin in 50% of lesions with varying degrees of tumour necrosis and lymphocytic infiltration (Fig. 5.4). Skin papillomas were observed in 27% of skin lesions; the remaining 23% mice showed various degrees of hyperplasia and dysplasia.

5.3.2 Epidermal LDC numeration in tobacco smoke condensate (TSC) treated skin

During the first two weeks there was an increase in the density of the Ia positive LDC in the skin of mice treated with TSC and the highest LDC level was achieved 28 weeks after treatment. The number of LDC in the epidermal sheets of the treated mice during this period was significantly higher (1793 LDC/mm^2) than that of the control group (946 LDC/mm^2), $p < 0.0001$ (Fig. 5.1).

At 45 weeks, almost 10 weeks after stopping the treatment with TSC, the number of LDC in the skin tumours (Fig. 5.5) and around lesions (Table 5.1) had not decreased, but in general remained elevated (Fig. 5.1). The

Figure 5.2a Multiple skin tumours after 35 weeks of TSC treatment (Scale in mm)

Figure 5.2b Skin tumours at 45 weeks, 10 weeks after stopping TSC treatment (Scale in mm)

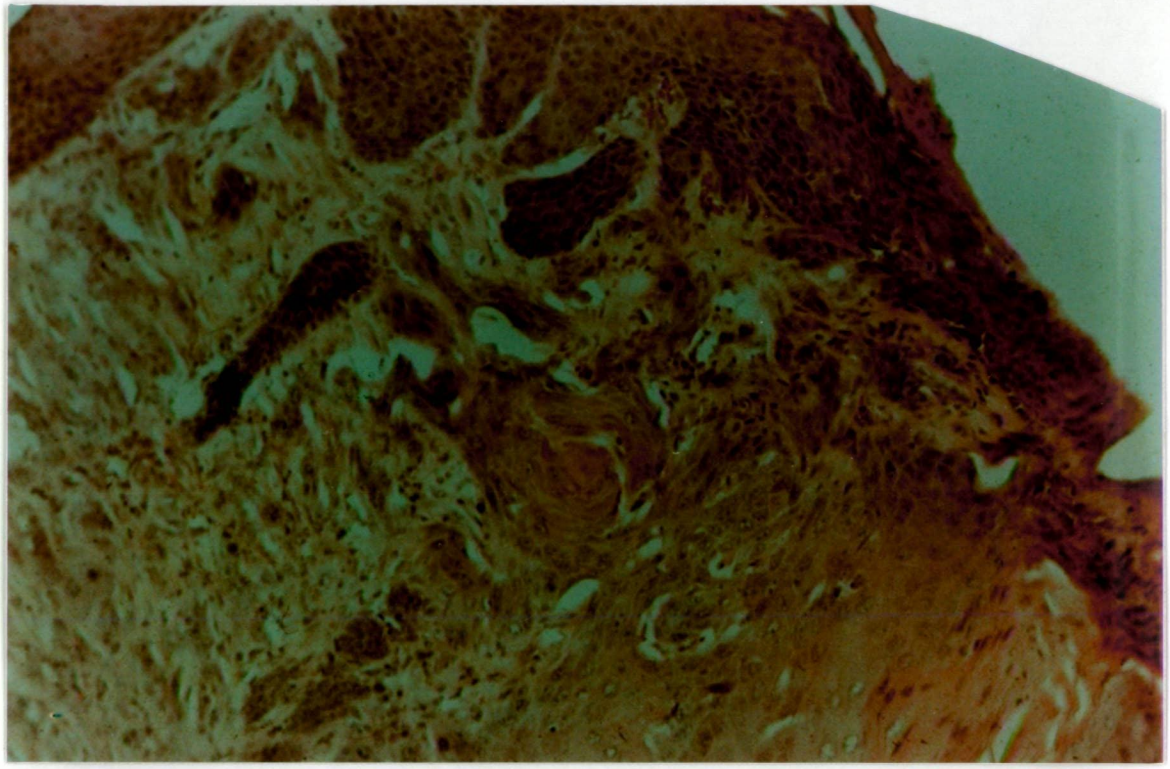


Figure 5.3 Invasive squamous cell carcinoma with mixed cellular reaction around tumour, 35 weeks after TSC treatment (H&E X 200)

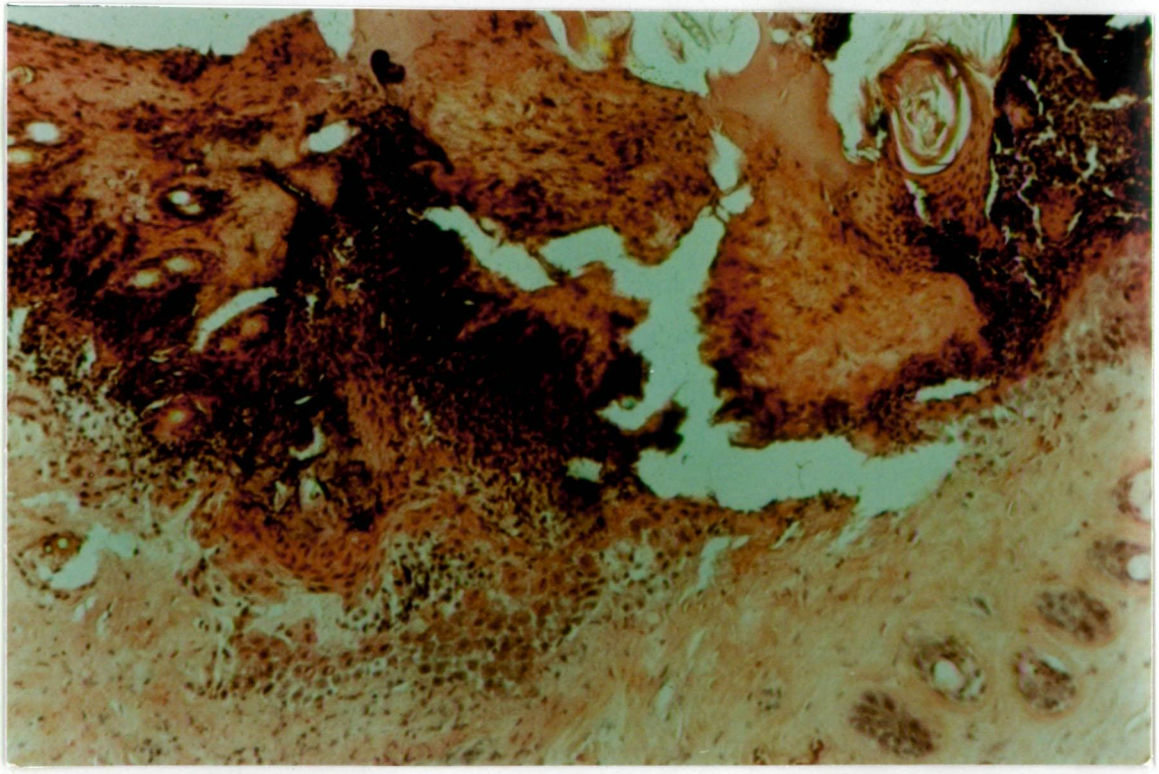


Figure 5.4 Necrotic squamous cell carcinoma of skin tumours at 45 weeks 10 weeks after stopping TSC treatment.(H&E X200)

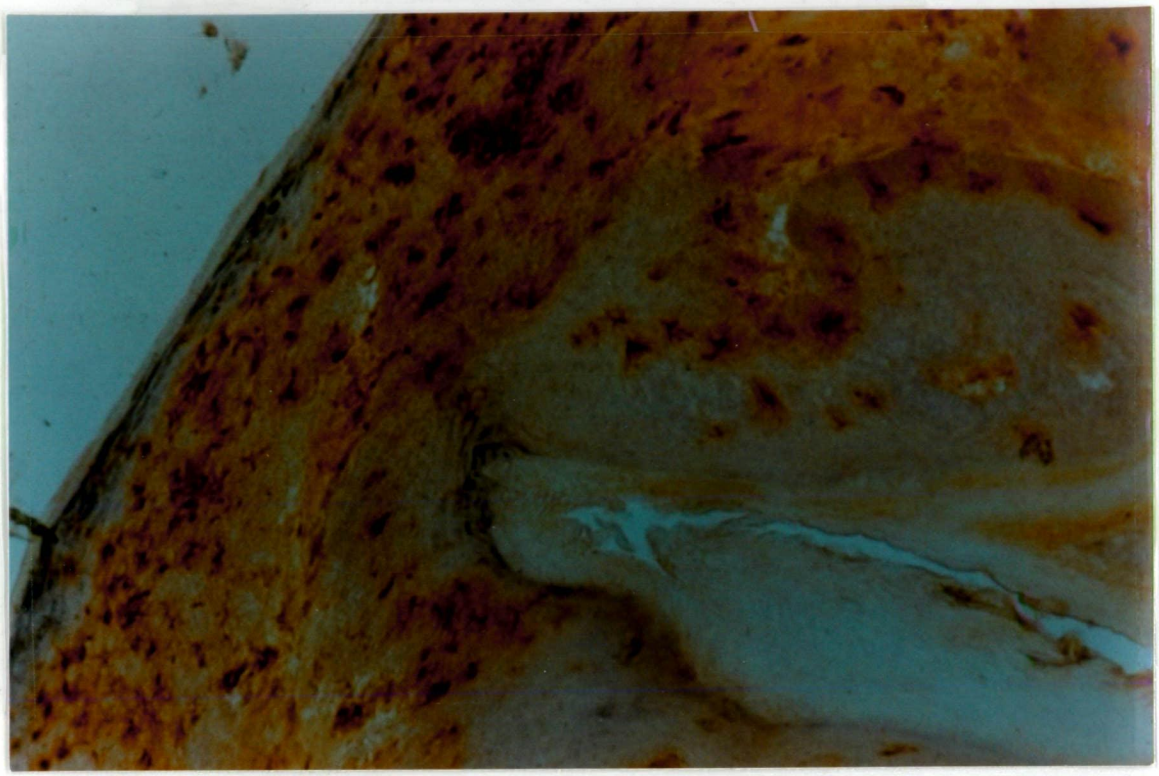


Figure 5.5 Ia positive LC in frozen section of skin tumour (Immunoperoxidase anti-Ia staining X 200)

Table 5.1 LDC around skin lesions 10 weeks after stopping TSC treatment

Skin lesion	No	Mean of LDC/mm^2	SD
1. Skin papilloma	13	2274	14.1
2. Invasive SCC	13	2088	183.0
3. SCC with necrosis	11	1960	61.3
4. Hyperplasia & dysplasia	11	1650	153.5

Using Scheffe's test at the 0.05 level, 2 versus (vs) 1, 3 vs 1, 1 vs 2 and 1 vs 3 no significant difference, While 4 vs 1, 2, &3 all significant.

exceptions were hyperplastic and dysplastic lesions. The difference in LDC number before and after stopping TSC treatment was significant ($p < 0.0001$).

5.3.3 LDC morphology in tobacco smoke treated skin

After 2 weeks of TSC treatment 25% of LDC in epidermal sheets became smaller in size and the dendrites were blunt and short compared with normal LDC (Fig. 5.6a). By 6 weeks many of the LDC cells were rounded or oval in shape (Fig. 5.6b). When TSC ceased at 35 weeks, the morphological changes reversed, the LDC became more dendritic and with more than 3 branched dendrites (Fig. 5.7).

5.3.4 Contact sensitivity response after TSC skin treatment

Table. 5.2 summarises the percent increase in ear thickness of both TSC treated and control mice at 2 and 6 weeks following sensitization and challenge with TNCB.

The percent increase in thickness of the right ear of the TSC treated mice when challenged after sensitization with TNCB was significantly less than that of the control group ($p < 0.0001$), at both 24 and 48 hours. This suggests an impairment in contact sensitivity response in the TSC treated mice and indicates a compromised function of the morphologically altered LDC.

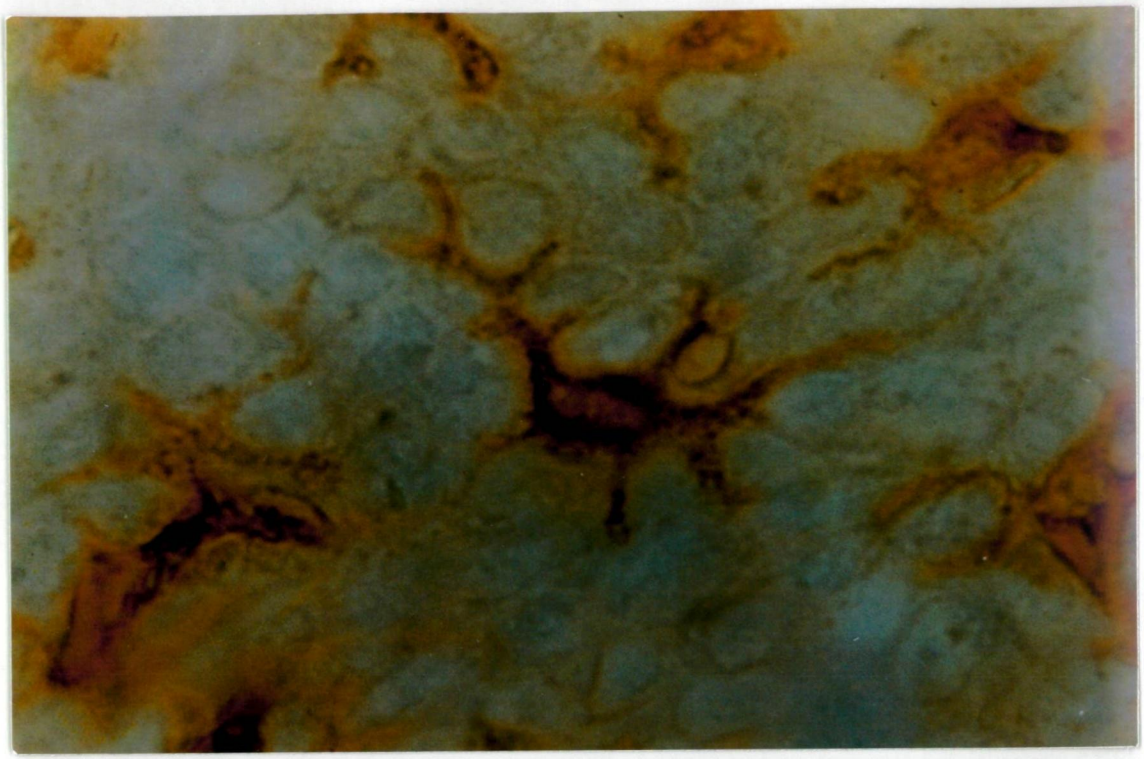


Figure 5.6a Normal fully dendritic LC in epidermal sheet of control mice (Immunoperoxidase anti-Ia staining X 1000)

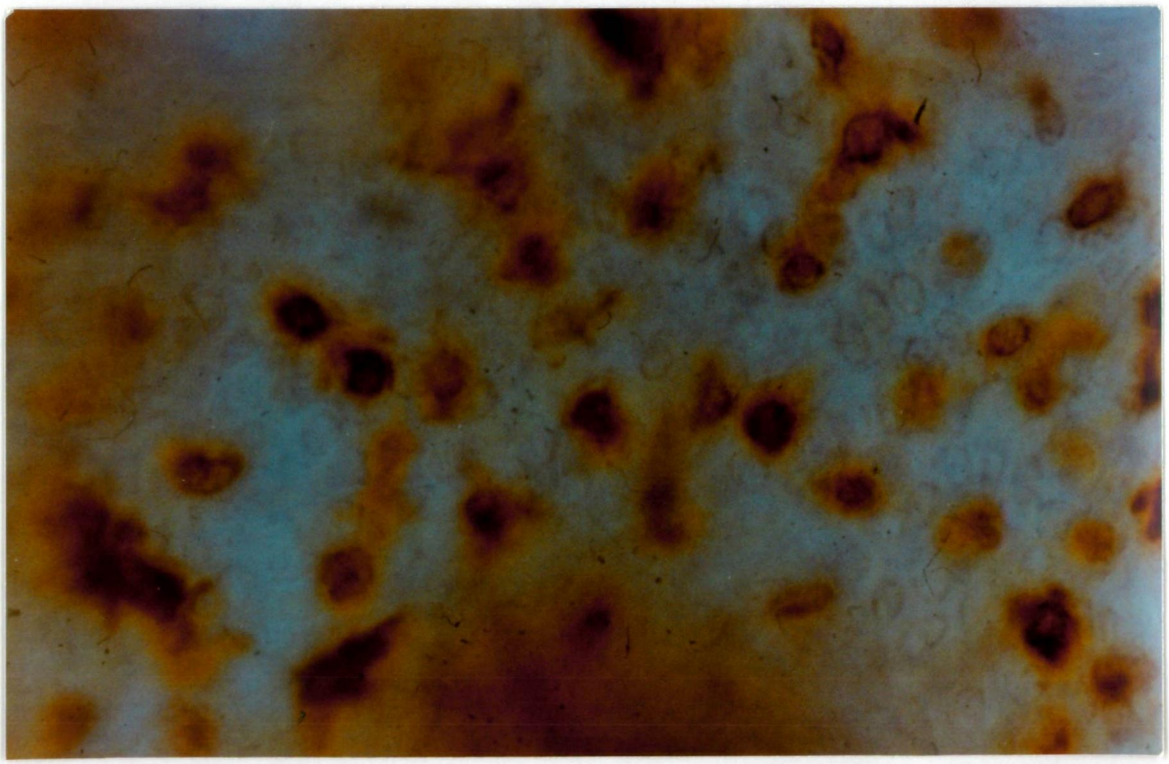


Figure 5.6b Rounded Langerhans Cells after TSC treatment (Immunoperoxidase anti-Ia staining X 400)

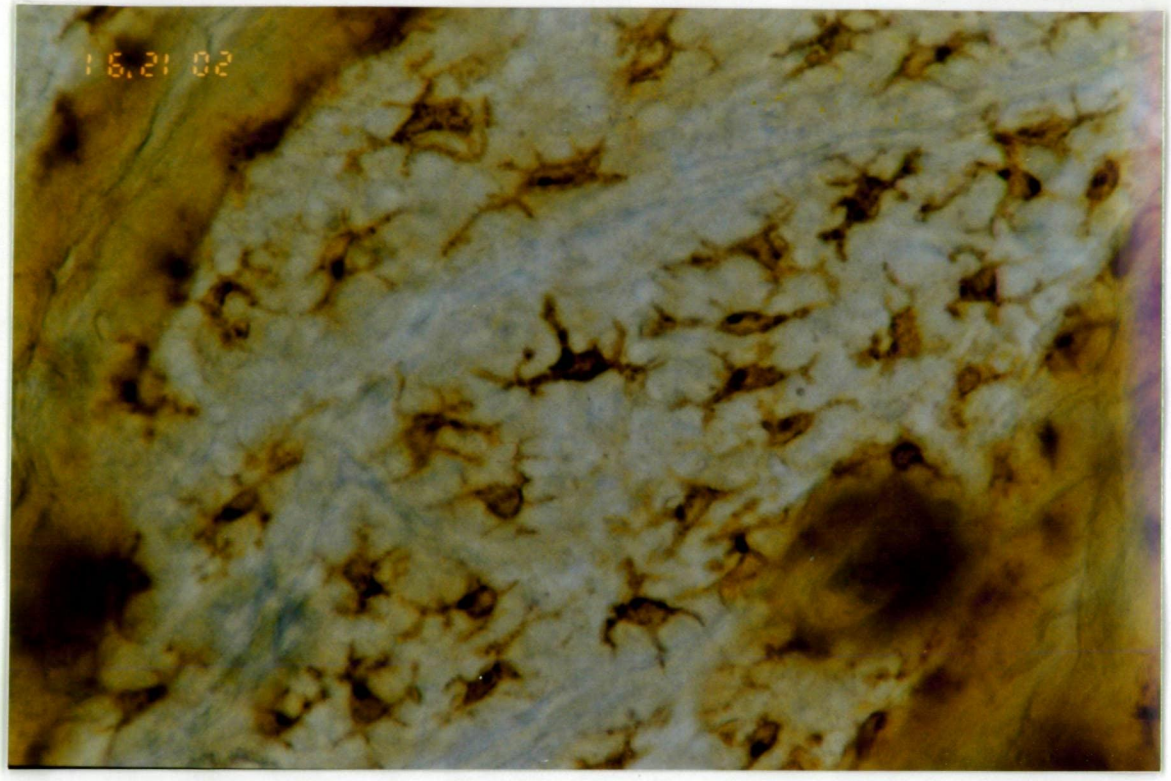


Figure 5.7 Langerhans Cells returned to their dendritic morphology, with more than 3 branched dendrites 10 weeks after stopping TSC treatment, some LC still abnormal in shape (Immunoperoxidaseanti-Ia staining X 400)

Table 5.2 Cutaneous contact sensitivity response to TNCB of mice treated with TSC

After 2 weeks

Group	No	Time	Mean of % increase in ear thickness
Control	6	24 HR	110 ± 19.96
TSC	6	24 HR	54 ± 9.95
Control	6	48 HR	81 ± 6.96
TSC	6	48 HR	42 ± 8.06

After 6 weeks

Control	6	24 HR	105 ± 6.59
TSC	6	24 HR	54 ± 9.95
Control	6	48 HR	76 ± 7.07
TSC	6	48 HR	50 ± 7.0

Differences between the % increase in ear thickness of test and control group is significant ($p < 0.0001$) at all times (Student's two-tailed, unpaired t-test).

5.4 Discussion

TSC increased the density of the epidermal LDC in epidermal sheets; with highest level 28 weeks after treatment (Fig. 5-1). At this stage the density of the epidermal LDC in the treated skin was double the control value and the high level of the epidermal LDC around lesions remained elevated even after stopping the TSC treatment. The morphology of epidermal LDC in the treated skin was also altered, as the cells were smaller and the dendrites became shorter and blunt. This was reflected in their function which was impaired as assessed by the contact sensitivity response. The initial increase in epidermal LDC number, the morphological changes and the impairment in their function observed during TSC treatment were also associated with tumour development. These findings support similar observations reported by Ruby et al., (1989) and Andrews et al., (1991) when they investigated the effects of benzo(a)pyrene on murine epidermal LDC. Andrews et al., (1991) demonstrated that implantation of the prostaglandin synthetase inhibitor indomethacin, beneath mouse skin before treatment with the tobacco derived carcinogen, benzo(a)pyrene delayed the onset of tumour development and reduced tumour size. Andrews and Co-workers, (1991) proposed that the cutaneous carcinogenesis of benzo(a)pyrene may be related to the suppression of the cellular cutaneous immunity induced by prostaglandins.

It is of interest that while the density of epidermal LDC decreased (1650 ± 154 cells/mm^2) in the skin of mice with hyperplastic and dysplastic lesions ten weeks after stopping the treatment with TSC, the density

increased, in both squamous cell papillomas (2273 ± cells/mm^2) and squamous cell carcinoma (2088 ± cells/mm^2). These LDC appeared fully dendritic and this increase was associated with spontaneous regression of 23% of skin tumours and 50% reduction in tumour size of remaining lesions. The high density of the fully dendritic epidermal LDC after stopping TSC application was accompanied by an increased LDC number in frozen sections of skin tumours. This was associated with marked lymphocytic infiltration and necrosis of these lesions.

Muller et al., 1985 also reported skin tumour regression after cessation of further application of the chemical carcinogen, 7,12-dimethylbenz (a) anthracene (DMBA). DMBA depleted epidermal LDC this was associated with impaired skin immune function and skin tumour development (Muller et al., 1992). Repopulation of epidermal LDC was associated with tumour regression (Muller et al., 1985).

The increase in LDC density in epidermal sheets around tobacco smoke induced squamous cell carcinomas (Table. 5.1) of the skin is consistent with the previous observations described in Chapter 3 that demonstrated an increase in $S100^+$ LDC in squamous cell carcinomas of the lung (Chapter 3). This is also in agreement with the results of previous workers including McArdle et al., (1986) and Korenberg et al., (1988) who reported an increase in LDC density in squamous cell carcinomas of the skin. Caorsi and Figueroa together with Bonilla-Musoles et al., (1987) found a similar LDC elevation in cervical squamous cell carcinoma as did Matsuda et al., (1990) who investigated squamous cell carcinoma of the oesophagus.

Langerhans dendritic cells are the best known antigen presenting cells and consequently their increase in certain tumours may follow a recognition by the LDC of a specific antigen expressed by tumour cells. Kato et al., (1977, 1979) reported a serum tumour antigen (Ta-4) in patients with cervical squamous cell carcinoma and suggested the serum level of the antigen was correlated with the disease progress. Yagi et al., (1987) also reported squamous cell carcinoma (SCC)-related antigen (SCC-RAG) in the serum of patients with cutaneous SCC. The titers of SCC-RAG was correlated tumour behaviours and with the development of metastasis. Grabbe et al., (1991; 1992) reported Ia^+ LDC are capable of presenting tumour associated antigen derived from a murine spindle cell tumour inducing an anti-tumour immune response manifested by tumour rejection and induction of delayed-type hypersensitivity in immunised animals.

While the extent of LDC infiltration of cutaneous and other tumours may be linked to tumour antigens, how LDC arrive in the skin lesions remains unclear. One possibility is that skin tumour derived cytokines attract LDC into the skin lesions (Halliday et al., 1992). Once functionally normal LDC are in lesions, they could trigger an immune response leading to tumour regression as in the present experiment.

It was concluded that TSC increased LDC number, altered their morphology, impaired their function; this was associated with tumour development. These events may be related to suppression of the cellular cutaneous immunity by prostaglandins as related to the effects of benzo(a)pyrene already described. The further increase in epidermal LDC

number after stopping TSC treatment was associated with reversible changes in LDC morphology, the cells became fully dendritic. These changes correlated with lymphocytic infiltration, tumour necrosis, reduction in tumour size and/or tumour regression presumably due to effective anti-tumour response.

5.5 Summary

Tobacco smoke condensate was painted on the skin of BALB/c mice. It increased the density and changed the morphology of Langerhans dendritic cells (LDC). LDC number in epidermal sheets of treated mice was significantly higher (1793 LDC/mm^2) than that of the control (946 LDC/mm^2) group, $p < 0.0001$ and remained elevated for 35 weeks. LDC became less dendritic, smaller in size or even rounded in shape. The function of the morphologically altered LDC was impaired when assessed by the contact hypersensitivity response. These changes were associated with skin tumour development in all treated mice.

Ten weeks after stopping the TSC treatment, LDC number in skin tumours and in skin around these lesions had not decreased, but significantly increased ($p < 0.0001$). During this period tumour regression occurred in 23% of tumours; the remaining tumours showed a 50% reduction in size. At 45 weeks, the LDC number in epidermal sheets around skin papillomas was $2273 \pm 14.1/mm^2$ and in invasive squamous cell carcinomas was $2088 \pm 183/mm^2$. This was associated with reversible changes in LDC morphology, where cells became fully dendritic. This also correlated with lymphocytic infiltration into tumours, tumour necrosis,

reduction in tumour size and/or tumour regression. It is concluded that the influx of normal LDC into the skin tumours allowed the development of an immune response with tumour regression.

CHAPTER 6

PRECURSOR OF LANGERHANS DENDRITIC CELLS

6.1 Introduction

While the bone marrow origin of LDC has been confirmed in animals (Frelinger et al., , 1979; Katz et al., 1979) and in humans (Pelletier et al., 1984; Volc-Platzer et al., 1984), its stem cell is unknown and their lineage relationship to monocytes and lymphocytes is disputed.

6.1.1 Langerhans dendritic cells in bone marrow

Goordyal and Isaacson, (1985) identified a small number of CD1a and $S100^+$ cells in cultured human bone marrow cells. Gothelf and coworkers, (1986) fractioned bone marrow mononuclear cells on the basis of peanut agglutinin positivity and reported 20% of the PNA^+ cells co-expressed CD1a (cortical thymocytes and LDC marker), CD11 (mature monocytes), CD14 (myelomonocytes), and possessed both Fc and C3 receptors. The co-expression of the monocytes markers with the LDC CD1a marker led Gothelf's group, (1986) to regard monocytes as the precursors of LDC. Fraissinette and co-workers, (1988) examined cultured $CD1a^+$ cells from human bone marrow and reported the co-expression of CD14, CD33, CD4, HLA-DR, HLA-DP but no specific T-lymphocyte markers. On ultrastructural examination, these cells did not contain BG in their cytoplasm. Fraissinette and co-workers, (1988) considered that these cells promonocytes and on the bases of CD1a expression they

proposed that these cells may represent the bone marrow precursors of LDC.

After long term bone marrow culture, Luksch and colleagues, (1989) reported a small fraction of the cultured cells co-expressed CD1a, CD1c, CD4, CD11b and CD11c. Reid and co-workers (1990) reported two groups of cells in the cultured bone marrow cells. One group co-expressed HLA-DQ and CD1a and the second group co-expressed HLA-DR and CD4. They suggested a common progenitor for both LDC and monocytes-macrophages. All the above reports proposed monocytes as the bone marrow precursor cells for the LDC.

6.1.2 Langerhans dendritic cells in the peripheral blood

The $CD1a^+$ LDC represent less than 1% of human peripheral blood mononuclear cells (Van-Voorhis et al, 1982). Similar findings were also reported by Dezutter-Dambyant and colleagues, (1984) when they investigated the LDC isolated from the cord blood of the newborn. These cells were CD1a and HLA-DR positive, adhere to plastic and following exposure to CD1a, BG-like structures were observed in their cytoplasm when examined by electron microscopy. Therefor the ability to induce and observe BG-like structures provided strong evidence to LDC nature of those cells.

Other reports have suggested that LDC precursor in the peripheral blood may express both T and monocyte surface markers, since they expressed CD11 (mature monocytes), CD14 (myelomonocytes), CD4 (T cells subsets) and CD3 (T cells) receptors, (Fraissinette et al., 1988;

Luksch et al., 1989; Reid et al., 1990; Steinman, 1991). Identification the bone marrow stem cell of LDC and their lineage relation to T-lymphocytes and monocytes awaits further clarification (Jaffe, 1993).

6.1.3 S100 protein and Langerhans dendritic cells

There are two subunits of the S100 protein a and b (Isobe et al, 1983). The commercially available antibody to S100 protein used in the present study reacts with both the a and b subunits but is predominantly anti-ß S100 protein.

The cytoplasm of both epidermal LDC and interdigitating dendritic cells (IDC) in the T-zone of lymph nodes contain S100 protein (Kahn et al., 1983). Small $S100^+$ lymphocyte-like cells can be found in lymph nodes and spleen (Watanabe et al., 1983), these cells have been proposed to be the precursors of LDC. Further investigation of LDC, and $S100^+$ IDC by Tanaka, (1986) revealed that both cells were $HLA\text{-}DR^+$ and $CD1a^+$. Uccini and colleagues, (1987) investigated the $S100^+$ cells in lymph nodes, thymus, spleen and peripheral blood. Consequently they reported two types of $S100^+$ cells; large and small lymphocyte-like cells, but in the blood only small $S100^+$ lymphocyte-like cells were found. This group was unable to characterise the small $S100^+$ lymphocyte-like cells but they agreed with Watanabe and co-workers, (1983) that the $S100^+$ small lymphocyte-like cells in lymph nodes are the precursors of LDC.

The distribution of S100 protein (α and β) in the lymphoreticular system and in the blood was investigated by Takahashi and colleagues (1984, 1985, 1987) and reported $S100\beta^+$ cells in the blood co-expressed CD3 and

CD8, and consequently considered these S100 cells as lymphocytes. Sansoni et al., (1987) reported S100ß$^+$ cells in the peripheral blood co-expressed both CD8 and CD11b surface markers, but were negative for the T cell subset surface marker, 9.3. They considered these cells a subset of T suppressor lymphocytes. It is noteworthy that the ultrastructural description of the S100ß$^+$ cells in the blood which have been reported as lymphocytes by Takahashi and colleagues (1984, 1985, 1987), was consistent with that of lymphoid dendritic cells reported by Steinman and Cohn, (1973); Von Voorhis et al., (1982); Dezutter-Dambuyant et al., (1984, 1988); Schuler and Steinman, (1985); Gothelf et al., (1987) and Romani et al., (1989).

6.1.4 Ultrastructure of Langerhans dendritic cells

The first ultrastructural studies of the isolated LDC from the lymphoid organs was performed by Steinman and Cohn, (1973) who reported the electron lucent cytoplasm contains large mitochondria with well-developed cristae, smooth surfaced vesicles, multivesicular bodies, long microtubules but no obvious BG were observed. Von Voorhis et al., (1982); Dezutter-Dambuyant et al., (1984, 1988) Schuler and Steinman, (1985); Gothelf et al., (1987) and Romani et al., (1989) supported the ultrastructure description of LDC by Steinman and Cohn, (1973) and added additional details on the irregularity of the nuclei and indentation of the nuclear membrane. The cytoplasm of these LDC did not contain the azurophilic granules found in monocytes (Zucker-Franklin D., 1975), and was not granular as in lymphocytes and granulocytes (Zicca et al., 1981; Monahan et al. 1981).

While studying human lung tumours S100$^+$ cells were observed in the lumen of small blood vessels (Chapter 3). This phenomena was most commonly observed in lung tumours rich in S100$^+$ LDC (bronchioalveolar, well and moderately differentiated squamous cell carcinoma). These S100$^+$ cells were attached to the endothelium, between the endothelial cells, and in blood vessel walls.

The aim of this investigation was to examine the nature of these S100$^+$ cells found in the blood and to investigate their lineage relationship to lymphocytes, monocytes and to the S100$^+$ tumour infiltrating LDC .

6.2 Materials and methods

6.2.1 Blood specimens

See Section 2.25.

6.2.2 Metrizamide enrichment of LDC from the peripheral blood

Mononuclear cells of PBMC and DC fractions were collected from human blood and cytospins were prepared for immunocytochemical staining. Further details are listed in Section 2.28.

6.2.3 Immunocytochemical staining of blood cells

Buffy coats and the cytospins of the PBMC and metrizamide enriched LDC were fixed in a mixture of acetone:methanol:formaldehyde. Blood cells were stained using rabbit antibody to bovine S100 protein using the

protocol previously described in Sections 2.26, 2.27, 2.28, 2.29. Monoclonal mouse anti-human T-cell- CD3; CD4; CD8; CD14 and CD45 were used to characterise blood cells. $S100^+$ cells, lymphocytes and monocytes numbers were expressed as % of total PBMC.

6.2.4 Double immunocytochemical labelling of PBMC and LDC

Cytospins were fixed in a mixture of acetone:methanol:formaldehyde. After suppression of the endogenous peroxidase, cytospins were incubated with antibody to the S100 protein, and then with the diaminobenzidine (DAB) substrate to localise the S100 protein. The second surface antigen was demonstrated using an indirect alkaline phosphatase technique. Cytospins were incubated with mouse anti-human surface antibody (CD3, CD4, CD8, CD14, CD45). The reaction colour for the second antigen was developed after incubating the cytospins with Fast Blue substrate solution. Further details are listed in Section 2.30.

6.2.5 Phagocytosis assay of $S100^+$ cells in the peripheral blood

PBMC were incubated in small tubes with polystyrene latex beads at 37 °C for 30 minutes. The supernatant was discarded, cells were washed and cytospins were prepared and stained for S100 protein. Further details are given in Section 2.31.

6.2.6 Adherent and non-adherent PBMC

PBMC were suspended in RPMI-FCS solution and incubated in Petri dish. Cytospins were prepared from both non-adherent and adherent cell fractions and stained for S100 protein. Details are listed in Section 2.32

6.2.7 Olympus CUE-2 image analysis system

Using the image analysis system, the perimeter and volume of the $S100^+$ cells (n= 75 cells) in peripheral blood were recorded and compared with that of the $CD3^+$ lymphocytes (n= 55 cells) and $CD14^+$ monocytes (n=12 cells). The means and the standard deviations were calculated (Section 2.33).

6.2.8 Micrometer eyepiece analysis

Using the micrometer eyepiece (Olympus OSM-D4), the perimeter, diameter and volume of $S100^+$ cells (n= 200) in peripheral blood were recorded and compared with that of the $CD3^+$ lymphocytes (n= 401 cells) and $CD14^+$ monocytes (n= 399 cells). The means, medians and the standard deviations were calculated (Section 2.34).

6.2.9 Immunoelectron microscopy examination of PBMC $S100^+$ cells

Modification of the methodology described by Takahashi et al., (1981) was used for the immunoelectron microscopy examination of the $S100^+$ cells in PBMC (Section 2.35).

6.2.10 Specimens for normal spleens and bone marrow

Three, 3-4 μm sections were cut (S100, control and H&E staining) from each paraffin blocks of 7 normal bronchial lymph nodes, 8 normal bone marrows and 10 normal spleens were obtained from the files of the Department of Anatomical Pathology, Royal Hobart Hospital (Section 2.36).

6.3 Results

6.3.1 $S100^+$ cells in blood of normal subjects

In blood samples of 100 normal subjects investigated in this study, $S100^+$ cells (Fig. 6.1) formed only 0.09 ± 0.07 cells x 10^9 per litre of blood, while lymphocytes represented 2.0 ± 0.6 and monocytes 0.26 ± 0.1. Their number in the blood of males and females was not significantly different, but there was a slight decrease in the number of $S100^+$ cells in the blood of individuals above 55 year. This was demonstrated using a scatter plot and computer generated line-of-best-fit (Fig. 6.2). In contrast to the effect of age, lymphocytes and monocytes did not change with age while the total white cell count increased marginally.

6.3.2 Characterisation of the $S100^+$ cells in the peripheral blood

Phagocytosis: The $S100^+$ cells did not phagocytose latex beads (Fig. 6.3), while monocytes were clearly observed to take up the beads. In a previous study (Chapter 3), when $S100^+$ dendritic cells in human lung tumours were investigated, alveolar pulmonary macrophages were S100 negative.

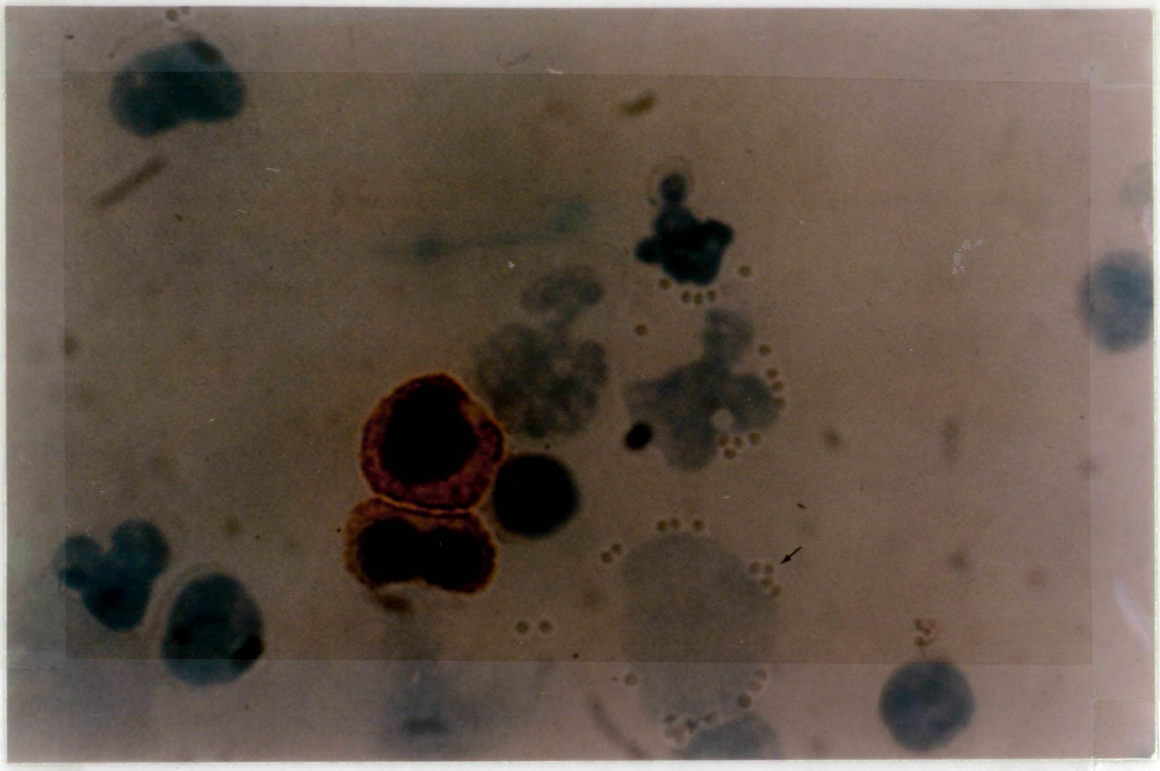


Figure 6.1 S100 positive dendritic cell in the buffy coats of PBMC (S100 Immunocytochemical Technique X 1000)

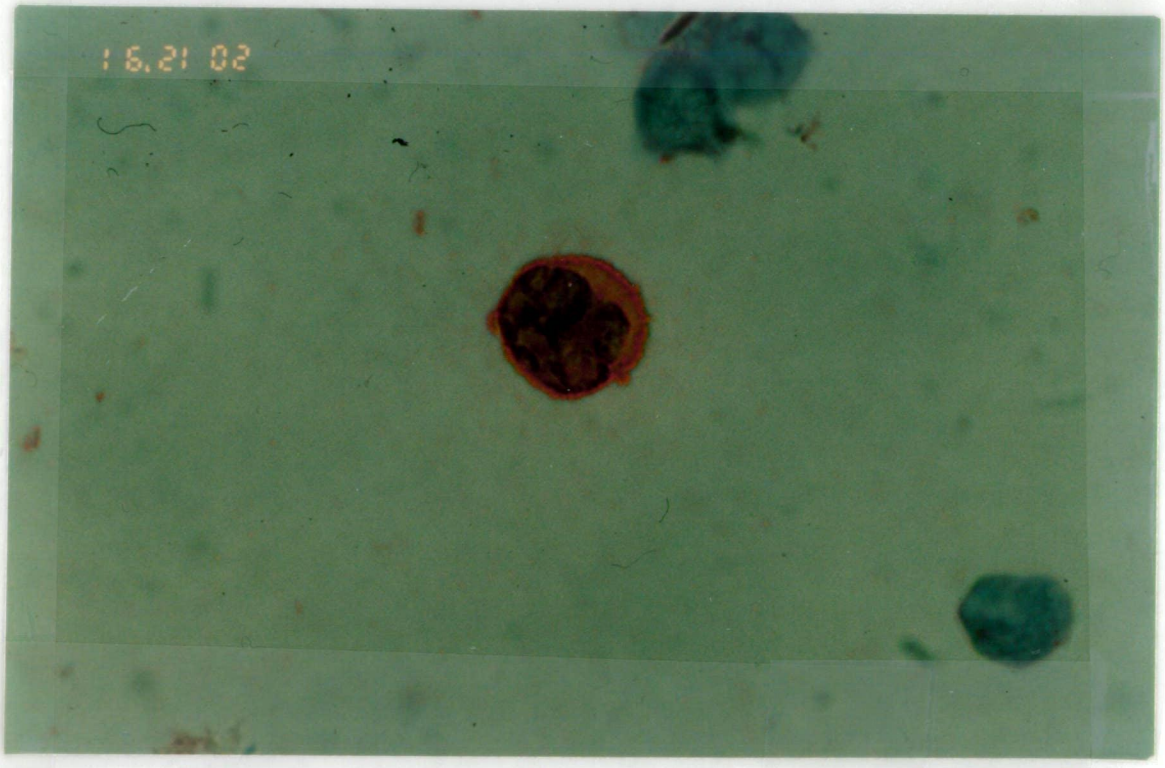


Figure 6.3 S100 positive dendritic cell were non-phagocytic (red cell), while monocytes phagocytosed latex beads (arrow)
(S100 Immunocytochemical Technique after incubation with latex beads X 1000)

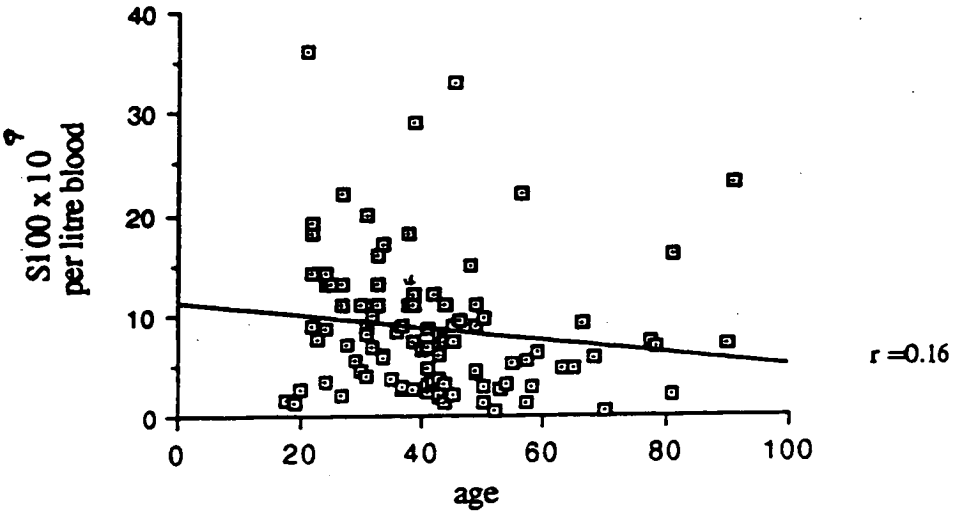


Figure 6.2 Effect of age on the number of S100+ cells per litre of blood

Adherence to plastic: When mononuclear cells were incubated in plastic petri dishes, 97.5% of $S100^+$ cells were found within the non-adherent fraction and only 2.5% within the adherent fraction.

Double immunolabelling: The $S100^+$ cells were assessed using monoclonal anti-bodies to CD45, CD4, CD3, and CD14. The co-expression of the cytoplasmic S100 protein and the surface antigens were difficult to determine as the surface antigen staining was always weak compared to the cytoplasmic S100 protein reaction. Only 1 ± 1% of the $S100^+$ cells in the blood co-expressed CD4, while 2.5 ± 1.40% co-expressed CD3 and 95 ± 5% co-expressed CD45. None of the $S100^+$ cells co-expressed CD14.

6.3.3 Metrizamide enriched Langerhans dendritic cells

The low density dendritic cells from the Ficoll-separated PBMC of human blood were collected and separated on metrizamide. The majority of metrizamide enriched dendritic cells isolated from human blood, were positive for S100 protein and CD45 surface antigen and did not express CD3, CD8 and CD14 (Fig. 6.4). Whereas the remaining cells collected from the pellet were $CD3^+$ with few $S100^+$ cells and there was no obvious co-expression of CD3 and S100 protein in any of the cells examined (Fig. 6.5).

6.3.4 Olympus CUE-2 image analysis system

The perimeter, volume, width and length of $S100^+$ cells, $CD3^+$ T lymphocytes and $CD14^+$ monocytes were analysed using the image analysis system. These parameters showed clear differences between the $S100^+$ cells, $CD3^+$ lymphocytes and $CD14^+$ monocytes ($p<0.001$). These

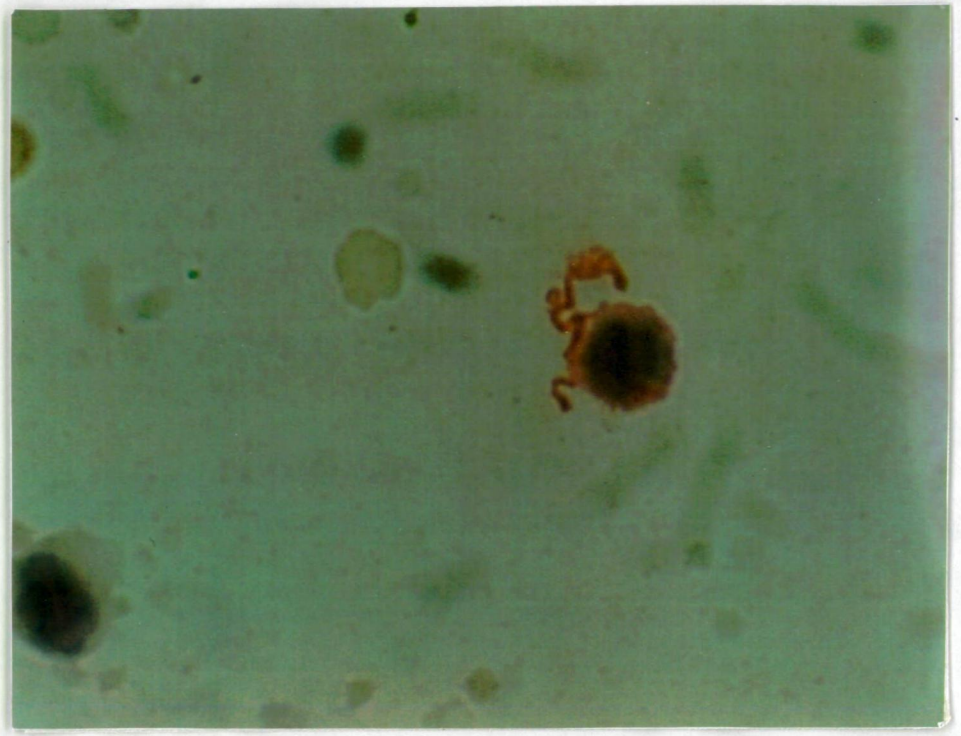


Figure 6.4 Metrizamide enriched DC are S100 positive (S100 Immunocytochemical Technique X 1000)

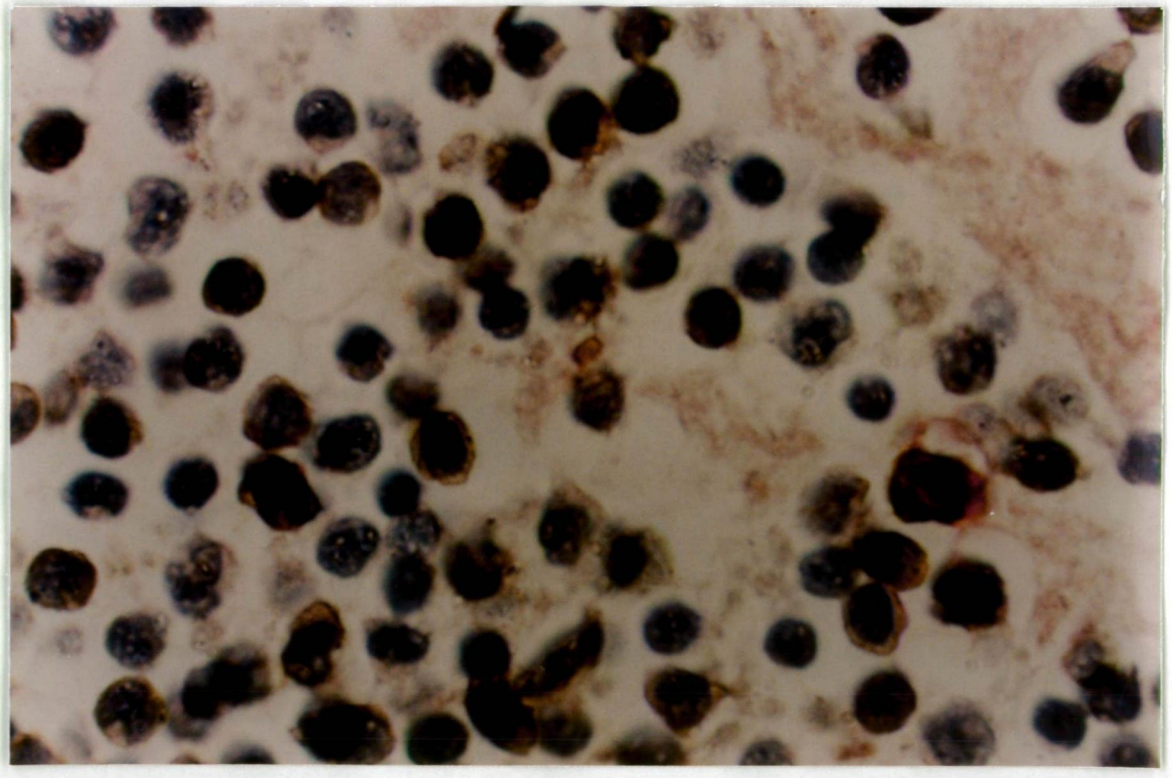


Figure 6.5 Double immunostaining of the mononuclear cell fraction after DC isolation. Majority of cells are CD3 + (black) and only one cell S100 positive (red). No obvious co-expression of CD3 and S100 (Double Immunocytochemical staining X 400)

results are listed in Table 6.1. The S100$^+$ cells are significantly larger than lymphocytes and smaller than monocytes.

6.3.5 Micrometer eyepiece analysis

The perimeter, diameter and volume of S100$^+$ cells, CD3$^+$ T lymphocytes and CD14$^+$ monocytes were analysed using the micrometer eyepiece. These parameters showed clear differences between the S100$^+$ cells, CD3$^+$ lymphocytes and CD14$^+$ monocytes ($p<0.001$). These results are listed in Table 6.2. The S100$^+$ cells are significantly larger than lymphocytes and smaller than monocytes.

6.3.6 Immunoelectron microscopy of the S100$^+$ cells in the peripheral blood

The peripheral blood S100$^+$ cells were identified with granular S100 protein scattered through the cytoplasm and around the nuclear membrane of these cells (Fig. 6.6a&b). Short dendrites extending from the cell membrane of these cells were demonstrated and were much shorter than those of epidermal Langerhans cells. The nuclei were large, frequently irregular and occasionally showed indentation of the nuclear membrane. A prominent rim of heterochromatin was consistently observed around the nucleus.

Although the cytoplasmic organelles were few, the mitochondria were numerous and large with prominent cisternae. No BG were found, but tubular structures, small single vesicles or multivesicular bodies with smooth limiting membranes were observed in the cytoplasm. The

Table 6.1 Image analysis of blood S100$^+$ cells, CD3 lymphocytes and CD14 monocytes

	S100$^+$ cells	CD3 lymphocytes	CD14 Monocytes
Number	75	55	12
Perimeter	26.3 ± 3.2	16.4 ± 1.7	33.1±3.4 ($p< 0.001$)
Length	8.7 ± 1.1	5.6 ± 0.61	11.3 ± 1.7 ($p<0.001$)
Width	7.1 ± 1.2	4.7 ± 0.61	7.3 ± 1.7 (*)
Volume	240.8 ± 64.5	61.6 ± ?	402.7 ± 63.43 ($p<0.001$)

The perimeter, length and volume were significantly different between S100+ cells, lymphocytes and monocytes ($p<0.0001$-0.001). While the difference in width between S100+ cells and lymphocytes was also significant ($p<0.001$), it was less significant between S100$^+$ cells and monocytes. (Measurements are in μm)

Table 6.2 Micrometer eyepiece measurements of blood $S100^+$ cells, CD3 lymphocytes and CD14 monocytes

	$S100^+$ cell	CD3 T lymphocytes	CD14 Monocytes
Number	200	401	399
Perimeter	31.37 ± 1.99	24.44 ± 3.43	41.24 ± 5.94 (p=0.0001)
Diameter	7.84 ± 0.5	6.1 ± 0.86	10.31 ± 1.49 (p=0.0001)
Volume	255.7 ± 49.49	126.75 ± 57.2	609.57 ± 258.57 (p<o.001)

The perimeter, diameter and volume were significantly different between S100+ cells, lymphocytes and monocytes (p<0.0001-0.001).
(Measurements are in μm)

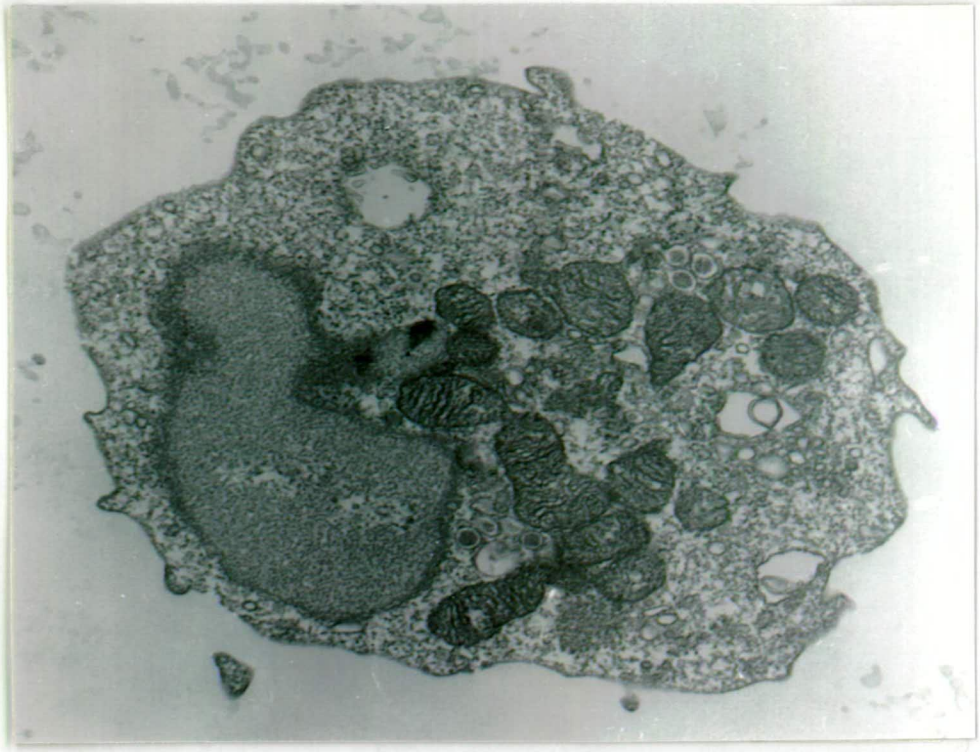


Figure 6.6a Micrograph of the S100 positive cell in blood: S100 reaction product in the cytoplasm and surrounds the nuclear membrane (Immunoelectron microscopy using immunoperoxidase Technique X 15000)

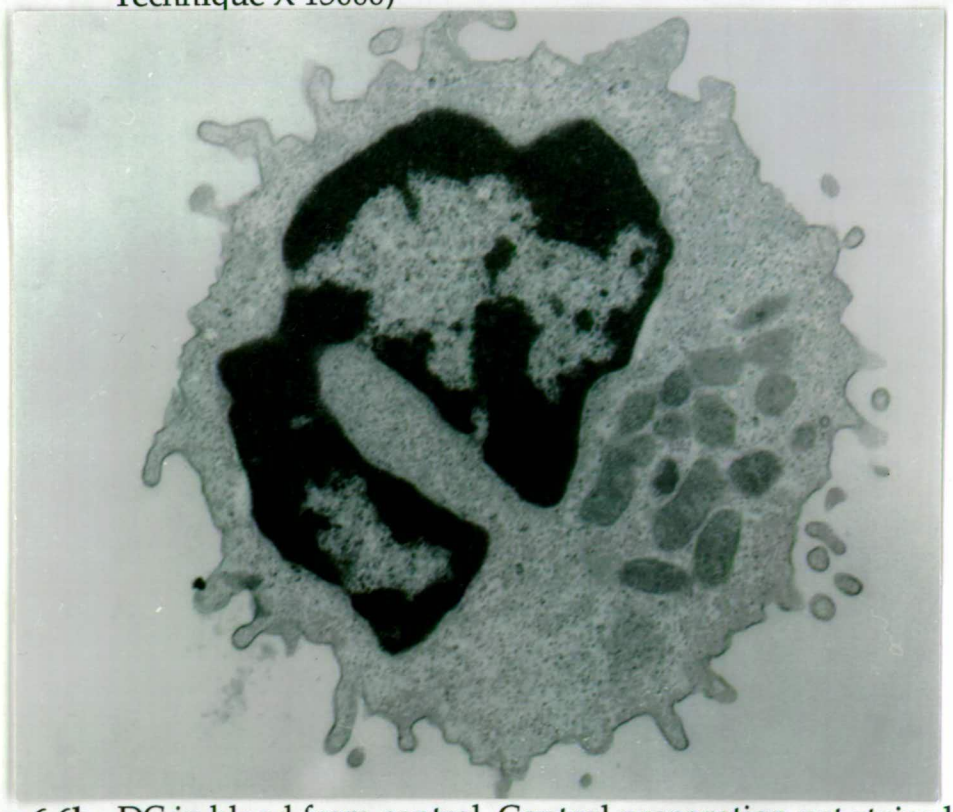


Figure 6.6b DC in blood from control, Control preparation not stained with anti-S100 antibody. The electrolucent cytoplasm contain numerous large mitochondria, tubular and vesicular structures (X 35000)

azurophilic granules of monocytes, lymphocytes and granulocytes were not observed in the cytoplasm of the S100+ cells.

6.3.7 S100+ cells in bone marrow of normal subjects

The S100+ cells were identified in all sections but with variable density, the mean of S100+ cells number was 3 ± 2 cells/2 HPF. The size and morphology of these cells varied, from small, oval or rounded cells. Some were without obvious dendrites on their surfaces while others were fully dendritic (Fig. 6.7).

6.3.8 S100+ cells in normal spleen

The S100+ cells were observed in the T-zone, mainly around the periarterial lymphoid sheaths (Fig. 6.8). Cells varied in size and morphology, small, intermediate and fully dendritic cells were observed. The mean of the S100+ cells number was 36.67 ± 17.67 cells/HPF. The density of S100+ DC and the range of their morphology in the spleen was almost similar to that in normal human lymph nodes (Fig. 6.9)

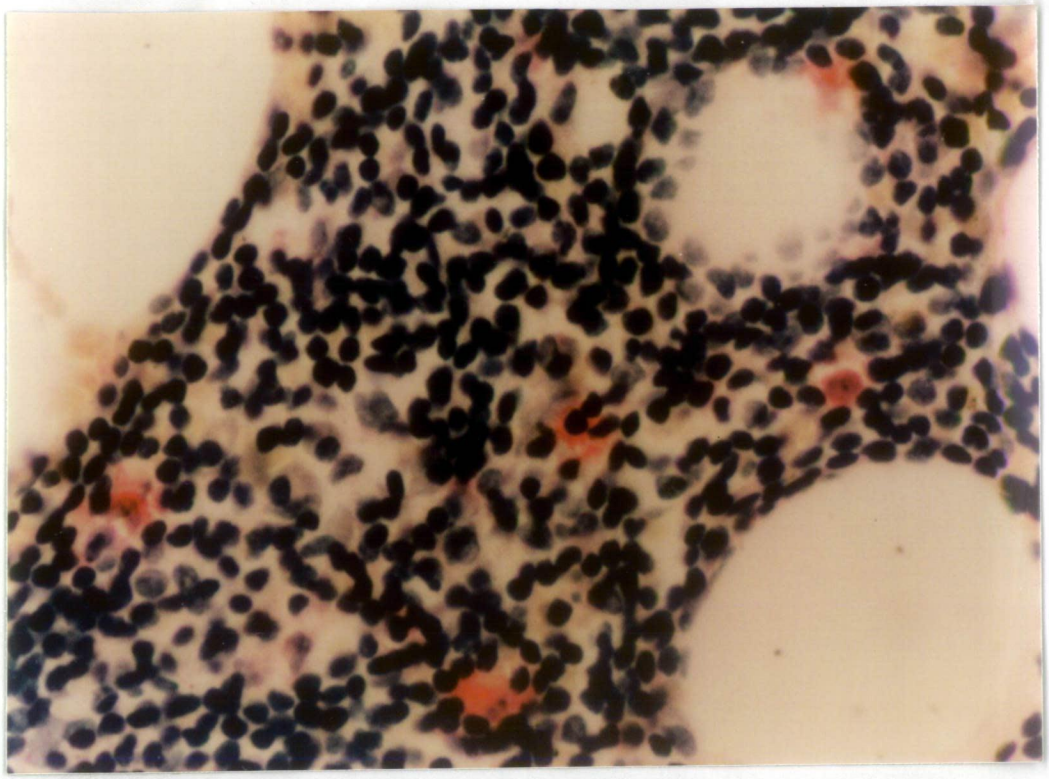


Figure 6.7 S100 positive DC with various morphology in normal bone marrow (S100 Immunohistochemical Technique X 200)

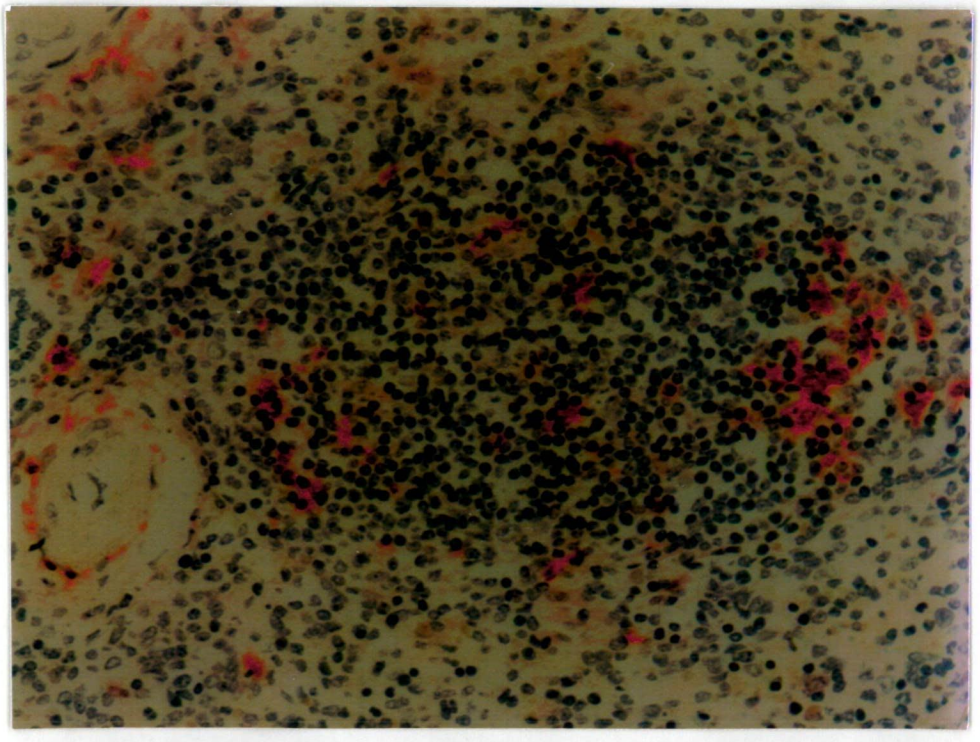


Figure 6.8 S100 positive DC of various morphology in the periarterial lymphoid sheaths of normal spleen
(S100 Immunohistochemical Technique X 200)

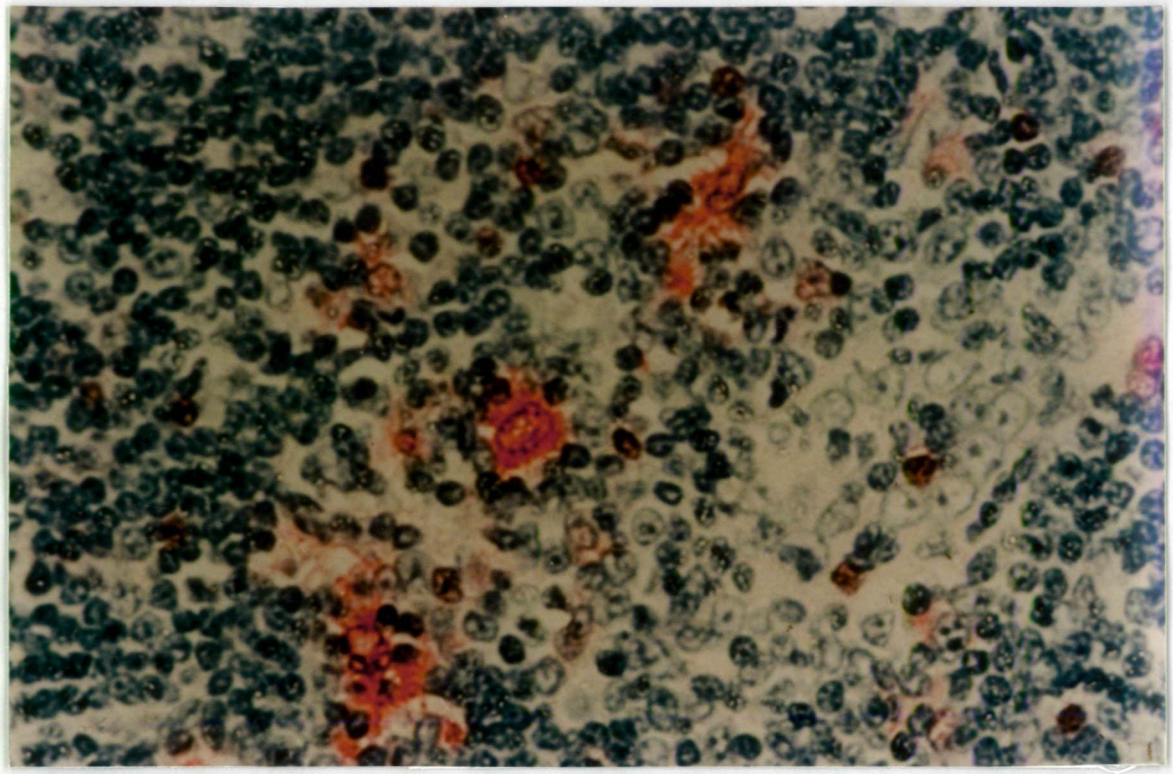


Figure 6.9 S100 positive DC, small, intermediate and fully dendritic in the T-zone of normal lymph node
(S100 Immunohistochemical Technique X 200)

6.4 Discussion

The bone marrow origin of LDC has been well established (Frelinger et al., 1979; Katz et al., 1979; Pelletier et al., 1984; Volc-Platzer et al., 1984), however the progenitor cell has yet to be confirmed as its lineage relationship to either monocytes or lymphocytes remains disputed (Jaffe, 1993). The cytoplasmic S100 protein is a strong and well accepted marker for both epidermal LDC and the interdigitating dendritic cells (IDC) found in the T-zone of lymph nodes (Kahn et al., 1983; Watanabe et al., 1983; Goordyal and Isaacson, 1985; Tanaka, 1986).

During the course of this study, $S100^+$ cells were identified in normal bone marrow, normal spleen and in normal lymph nodes. The size and morphology of these cells varied, some had obvious dendrites on their surfaces, whereas others were more rounded and have only short and blunt dendrites. However, in the peripheral blood, the $S100^+$ cells were always small and without prominent dendrites extending from their cell membrane. Such variation in size and morphology of LDC in tissues is consistent with the previous observations of the $S100^+$ LDC in various lung tumours (Zeid and Muller, 1993, Chapter 3) and in agreement with similar observations reported by both Watanabe et al., (1983) and Uccini et al., (1987).

The $S100^+$ cells in the blood, and the isolated metrizamide enriched dendritic cells, were found to be identical in both size and morphology as determined by image and micrometer eyepiece analysis. These cells, even though classified as small, were significantly larger than the $CD3^+$ lymphocytes ($p<0.001$) but smaller than the $CD14^+$ monocytes. Uccini and

colleagues (1987) investigated the S100+ LDC in lymph nodes, thymus, spleen and peripheral blood and described them either large dendritic or small lymphocyte-like cells. The detection of S100+ LDC in lymph nodes, thymus and spleen indicates a wide distribution of these cells and is consistent with the notion that LDC originate in the bone marrow and migrate through the blood to reach the target organs or tissues including the skin.

Only 0.09 ± 0.07 cells x 10^9 per litre of blood were S100+ compared to the majority of metrizamide enriched dendritic cells isolated from normal human blood. These S100+ cells were non-adherent to plastic, co-expressed CD45, did not phagocytose latex beads and did not express the monocyte surface marker, CD14. A small number of S100+ cells in the peripheral blood (2.5 ± 1.4%) co-expressed weak CD3. The majority of cells in the mononuclear cell fraction remaining after isolating the metrizamide enriched DC, were CD3+ with few S100+ cells and there was no obvious co-expression of both markers by any of the cells examined (Fig. 6.5). From these observations it is apparent that by purifying for dendritic cells you are enriching for S100+ cells, whereas purifying for T cells you are depleting S100+ cells. There is clear segregation of these cell types.

The ultrastructural examination of S100+ cells in the blood (Fig. 6.6) supports the concept that these S100+ cells are LDC but different from lymphocytes and monocytes. S100+ cells in the blood had a few short dendrites extending from the cell membrane, S100+ protein granules could be seen in the cytoplasm and around the nuclear membrane which was often seen with indentation. The translucent cytoplasm contained

numerous and large mitochondria, tubular cytoplasmic structures, single or multivesicular bodies, but no classical BG were seen. This description is totally consistent with the ultrastructure of LDC previously described (Von Voorhis et al., 1982; Dezutter-Dambuyant et al., 1984, 1988; Schuler and Steinman, 1985; Gothelf et al., 1987; Romani et al., 1989) and are clearly different from T lymphocytes (Zucker-Franklin, 1975; Zicca et al., 1981; Monahan et al. 1981).

Steinman and Cohn (1973) reported the ultrastructure of the isolated LDC from the lymphoid organs. They described these cells with electron lucent cytoplasm containing large mitochondria with well-developed cristae, smooth surfaced vesicles, multivesicular bodies, long microtubules but no obvious BG were observed. Irregularity of the nuclei and indentation of the nuclear membrane was also a common feature of these cells. This ultrastructural description has been repeatedly maintained by many workers (Von Voorhis et al., 1982; Dezutter-Dambuyant et al., 1984, 1988; Schuler and Steinman, 1985; Gothelf et al., 1987; Romani et al., 1989). The cytoplasm of these LDC did not contain the azurophilic granules found in monocytes (Zucker-Franklin, 1975) and was not granular as in lymphocytes and granulocytes (Zicca et al., 1981; Monahan et al., 1981).

Based on these observations it is proposed that these $S100^+$ cells in the blood represent the precursor of LDC found in human skin, spleen and lymphoid tissue. The findings in this study support the observations of Watanabe et al., (1984) and Uccini et al., (1987) as they reported a lineage relationship between the $S100^+$ LDC and $S100^+$ lymphocyte-like cells in the blood, lymph nodes and spleen.

In contrast Takahashi and co-workers (1984, 1985, 1987); De Panfilis et al., (1988); Sansoni et al., (1987); Ferari et al., (1988) who reported that S100$^+$ cells in blood as T lymphocytes, did not undertake image analysis of these cells and did not compare their parameters with T lymphocytes or monocytes. Their findings were based on the results of double immunostaining of S100$^+$ cells in the blood. They claimed that a small fraction of CD3$^+$, CD8$^+$ and CD11b$^+$ lymphocytes in the blood co-expressed the cytoplasmic S100ß protein. In a subsequent report these workers appear to have contradicted themselves by, in the first instance, describing T cells on the basis of CD11 expression, and then describing LDC as expressing CD11b (De Panfilis et al., 1988). This antigen is not unique to T cells and in fact De Panfilis et al., (1988) described epidermal LDC on the bases of their expression of CD11b and CD11c in combination with CD18 and S100ß and therefore the CD11b expressing cells from the earlier reports could have been either T cells or LDC. Double immunostaining of surface and cytoplasmic antigens is not always conclusive, because of the need of various fixatives to demonstrate the surface antigen and the cytoplasmic S100 protein. The difference in colours of the reaction products between the strong cytoplasmic S100 protein and the weak surface antigen is difficult to distinguish. Uccini et al., (1987) reported difficulty when they tried to double immunostain the S100$^+$ small cells they observed in blood, spleen and lymph nodes with T or monocyte surface markers. From the work described in this study only a small number (2.5 ± 1.4%) of S100$^+$ cells in the peripheral blood coexpressed weak CD3 antigens. The majority of cells in the mononuclear cell fraction remaining after isolating the metrizamide enriched LDC, were CD3$^+$ with only few remaining S100$^+$ cells and there was no obvious co-expression of both markers by any of the cells examined.

The ultrastructural description of the S100ß positive cells in the blood (Takahashi et al., 1985, 1987; Sansoni et al., 1987; De Panfilis et al., 1988; Ferari et al., 1988) is actually consistent with that of blood and lymphoid dendritic cells (Von Voorhis et al., 1982; Dezutter-Dambuyant et al., 1984, 1988; Schuler and Steinman, 1985; Gothelf et al., 1987; Romani et al., 1989). Based on the evidence presented in this study, it is likely that the $S100^+$ lymphocytes described in the blood are more likely to be $S100^+$ dendritic cells than T lymphocytes.

Since 1989, only one report has appeared describing $S100^+$ T cells (Hanson et al., 1991). This group described 4 cases of chronic lymphoproliferative disease of an uncommon $S100^+$ T cell. Close analysis of the above report revealed many contradictory results. While Hanson et al., (1991) reported that the leukemic cells in the blood were $CD3^+$ and therefore considered them as T cells, they failed to clarify if these leukemic cells express the cytoplasmic S100 protein. It was also unclear in this report if the $CD3^+$ cells coexpressed S100 as double labelling experiments were not performed. Furthermore, at no time did the author present data on the percent of $S100^+$ cells and therefore there is no clear evidence whether the TCR DNA and RNA gene studies of the leukemic cells were carried out on the $S100^+$ cells, or in fact the $CD3^+$ cells. Hanson et al., reported that the leukemic cells had no cytoplasmic granules, but subsequently contradicted themselves when they described them with occasional electron-dense cytoplasmic granules following ultrastructural examination. It was not clear from the ultrastructural description of the leukemic cells, whether they examined the $CD3^+$ cells, or the $S100^+$ cells. The best evidence for T cell origin is TCR gene rearrangement. This was performed on the leukemic cells, but again, on further analysis, there was

no evidence that these cells were $S100^+$. It is most likely that these workers detected some $S100^+$ cells but there is no evidence that these cells were the described leukemic cells. It is most likely that their description was of the leukemic cells which were possibly NK cells as they killed K562 cell and expressed CD56 and CD16.

It is noteworthy to observe that when other workers investigated the $S100^+$ LDC and other lymphoid cells in various human tissues, there has not been a single report describing T lymphocytes coexpressing S100 protein in their cytoplasm (Timens, et al., 1988; Temenia et al., 1990; Huang, 1990; Gallo et al., 1991; Cribier, 1992; Shin and Co-workers, 1992, Yamaguchi et al., 1992; Gaillard et al., 1993; Colasante et al., 1993; Paschke et al., 1993; Chaux's group, 1993; Hart and Co-workers, 1993). Though, dendritic and other blood cells may share the same bone marrow stem cell, they differentiate as a separate cell line. LDC may originate from a multilineage haematopoietic stem cells in the bone marrow (Fleming et al., 1993; Furuta et al., 1993) and express CD45 and some T cell surface markers at various stages of maturation. The fact that these antigen presenting LDC specifically stimulate T lymphocytes, the expression of some T lymphocytes surface antigens may be required in the process of recognition and stimulation of the resting T lymphocytes in the lymphoid tissue. Shedding some of the cell surface molecules by the activated T lymphocytes has been reported (Dooley et al., 1993), such molecules may be picked up by LDC during the immunological interaction between the antigen presenting LDC and T cells. Subsequently these LDC may appear to express some of the T cells surface molecules such as CD3.

Based on the evidences presented in this study and supported by the findings of various workers mentioned above, it is concluded that the small $S100^+$ cells found in the peripheral blood, represent the precursors of LDC found in human skin, bone marrow, spleen, lymph nodes and some pathological conditions such as tumours. While they co-express the common leukocyte surface marker CD45 and occasionally weak T cell surface markers, there is no obvious lineage relationship with either T lymphocytes or monocytes.

6.5 Summary

Metrizamide enriched Langerhans dendritic cells (LDC) isolated from human blood, were $S100^+$, $CD45^+$, $CD3^-$, $CD8^-$, $CD14^-$ and did not phagocytose latex beads. The perimeter, diameter, width, length and volume of these $S100^+$ cells was larger than that of $CD3^+$ T lymphocytes and smaller than that of $CD14^+$ monocytes. Their ultrastructural features were consistent with that of Langerhans dendritic cells from the skin as the nuclei were large, indented with a prominent rim of heterochromatin around the nuclei and the mitochondria were numerous and large. While no Birbeck granules (BG) were observed, tubular structures, single vesicles and multivesicular bodies were present. Although lymphocytes represented 2.0 ± 0.6 and monocytes 0.26 ± 0.1, $S100^+$ dendritic cells represented only 0.09 ± 0.07 cells $\times 10^9$ per litre of blood. Equal numbers of blood $S100^+$ cells were observed in males and females, but their number slightly decreased with age. It was concluded that $S100^+$ blood mononuclear cells are of a lineage relationship with other $S100^+$ Langerhans dendritic cells found in human bone marrow, skin, spleen, lymphoid tissue and in human tumours. Although these

cells co-express the common leukocyte surface marker CD45, there was no obvious lineage relationship with T lymphocytes or monocytes.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

Tobacco smoke remains a major cause of lung tumours and chronic pulmonary diseases. By early 1980 more than half a million people in the USA were dying each year from diseases that were directly related to smoking (A Report of the Surgeon General, 1982). The annual cost of medical care for smoking related illnesses in Canada and USA is in excess of US $ 501 billion (A 1992 report of the Surgeon General). In West Australia, 13,944 deaths were a result of tobacco smoking between 1981-1990 (Deaths from tobacco smoking in Western Australia: 1981-1990, WA Drug Data Collection Unit, 1992).

Tobacco smoke contains seventy one carcinogens and co-carcinogens (Bos and Henderson, 1984), the concentration of these in sidestream tobacco smoke is higher than in the mainstream tobacco smoke (Hoegg, 1972). This highlights the dangers of passive exposure to tobacco smoke as not only are the smokers at risk of lung cancer but individuals passively exposed to tobacco smoke may also be affected. This was confirmed when Hirayama, (1981) investigated 91,540 non-smoking married women for 14 years and reported that wives of heavy smokers have a higher risk of developing lung cancer than wives of non-smokers. Previously Casolaro, (1988) and Soler et al., (1989) reported that tobacco smoke increases the density of pulmonary LDC, but to date the effects of tobacco smoke on the density and

function of the pulmonary antigen presenting LDC and their role in tobacco smoke induced lung lesions and lung tumours have not been fully investigated.

The first part of this study (Chapter 3) investigated the density of LDC infiltrating tobacco smoke related human lung cancer and in the draining lymph nodes to determine if there was a relationship between LDC density and prognosis. Furthermore an analysis of tumour infiltrating lymphocytes was undertaken to ascertain the interaction of LDC and TIL with patient survival and prognosis. The observations from the human lung study led to the conclusion that there was a correlation between tumour type, LDC and TIL density which was a direct consequence of exposure to tobacco smoke and its carcinogens. This led to next phase of the project which included the establishment of an animal model to examine more directly the effect of tobacco smoke and its condensate on LDC. The effect of passive tobacco smoke inhalation on murine pulmonary LDC investigated in Chapter 4, and furthermore the effect of tobacco smoke condensate on murine epidermal LDC during cutaneous carcinogenesis was examined in Chapter 5.

During the analysis of LDC in human lung tumours, cells stained for S100 were observed within the blood vessels. Upon further analysis these $S100^+$ cells were detected extravasating from the blood vessels into the perivascular lymphoid tissue. This raised the important question regarding the lineage relationship between these $S100^+$ cells found in the blood vessel lumen and the $S100^+$ tumour infiltrating LDC. In Chapter 6

the nature of the S100$^+$ cells in the blood was investigated and their lineage relationship to lymphocytes, monocytes and LDC analysed.

7.2 Langerhans dendritic cells and tobacco smoke related human lung tumours

Immunohistochemical analysis of human lung tumours clearly demonstrated that fully dendritic S100$^+$ LDC were often in direct contact with tumour cells and T lymphocytes. A direct relationship between the number of LDC and the density of TIL was observed in specific tumour types including bronchioalveolar, well and moderately differentiated squamous cell carcinomas. This was associated with central tumour necrosis and increased patient survival. Such observations were not as marked in the human lung tumours where the number of tumour infiltrating LDC was low and patient survival less favourable e.g. poorly differentiated squamous cell carcinoma and SCLC. Consequently the increased number of fully dendritic tumour infiltrating LDC and the associated TIL was associated with tumour necrosis and longer patient survival. This is indicative of an active immunological interaction between certain tumours, LDC and TIL. This is supported by the observations of Caorsi & Figueroa, (1984) and Mooris et al., (1983). They also reported an increase in the number of fully dendritic LDC in squamous cell carcinoma of the cervix and this was associated with marked TIL. They proposed that this might indicate a specific immune response against the neoplastic cells. Such a condition is also supported by Xie, (1990) who reported an increased density of S100$^+$ Langerhans

dendritic cells in cervical carcinoma and this was associated with longer patient survival.

Why bronchioalveolar, well and moderately differentiated squamous cell carcinomas were infiltrated with a greater number of LDC than other tumours e.g. SCLC and poorly differentiated squamous cell carcinoma is an important question. One possible explanation is that the LDC are attracted to the tumour due to the presence of a tumour antigen. Evidence for such antigens has been reported for bronchioalveolar, well and moderately differentiated squamous carcinoma (Faber, 1941; Kato et al., 1977; 1979; Dairaku et al., 1983; Yagi et al., 1987; Katyal and Kawai et al., 1988; Hance, et al., 1988). These antigens could be recognised by the LDC which could be transiently retained in the tumour and attract further LDC through the elaboration of cytokines which further increased their number and TIL density in these tumours. The ability of LDC to present tumour associated antigen has been investigated by Grabbe et al., (1991 & 1992) who found that LDC can present tumour associated antigen derived from a murine spindle cell tumour. This resulted in the induction of an anti-tumour immune response manifested by tumour rejection and induction of delayed-type hypersensitivity in immunised animals.

The collective evidence presented in this thesis supports the possibility that bronchioalveolar, well and moderately differentiated squamous cell carcinoma express a tumour associated antigen which the LDC could present to T lymphocyte resulting in their activation which leads to tumour necrosis and a more favourable prognosis. Such an antigen could

be absent or at least poorly expressed, in SCLC and poorly differentiated squamous cell carcinoma (Broers and Ramaekers, 1994) as these tumours do not appear to induce LDC infiltration.

7.3 Langerhans dendritic cells and tobacco smoke induced lung lesions

Exposure of mice to tobacco smoke in the absence of any other confounding factors such as environmental exposure to other carcinogens resulted in the development of pulmonary Langerhans cell granulomatosis, squamous metaplasia of the bronchial epithelium, alveolar and bronchial lesions (Chapter 4). This was associated with an increase in the number of pulmonary LDC. This is the first clearly defined experimental observation that directly links tobacco smoke exposure, LDC and the development of such a lesion. This relationship therefore implicates pulmonary Langerhans dendritic cells directly in the pathogenesis of pulmonary Langerhans cell granulomatosis.

The increase in the density of pulmonary LDC induced by tobacco smoke is supported by Casolaro, (1988) and Soler et al., (1989) who analysed and show their accumulation in smokers' lungs. The mechanism by which tobacco smoke increases the density of pulmonary LDC is not clearly understood. One possibility is that the LDC recognised some of the chemical compounds, including the 71 carcinogens (Dube and Green, 1982; Bos and Henderson, 1984) in tobacco smoke as an antigen (Woods et al., 1993; Ragg et al., 1994). Alternatively the damaged alveolar and bronchial epithelium induced by the tobacco smoke which may have expressed neoantigens (Faber, 1941; Kato et al., 1977; 1979; Singh, 1979;

Dairaku et al., 1983; Yagi et al., 1987; Katyal and Kawai et al., 1988; Hance, et al., 1988) which triggered the increase in LDC density as described in Chapter 4. It was also reported that benzo(a)pyrene and catechols of tobacco smoke increase the number and impair the LDC function by prostaglandin release (Muller et al., 1985; Odling et al., 1987; Ruby et al., 1989; McMinn et al., 1990, 1991; Andrews et al., 1991). This increase in the pulmonary LDC number could lead to T-lymphocyte activation (De Panfilis et al., 1989; Muller et al., 1994) and these sensitised T-lymphocytes would interact with the antigen bearing target cells, bronchial and alveolar epithelium, leading to their necrosis and inflammatory reaction and eventually interstitial granuloma formation.

When further exposure to tobacco smoke ceased for six weeks, the pulmonary LDC number in the lungs of the remaining mice was simillar to that of the control level. Furthermore, neither interstitial granulomas nor alveolar or bronchial hyperplastic-neoplastic lesions were observed. These changes further implicate tobacco smoke induced LDC changes in the pathogenesis of these pulmonary lesions and also highlights the significant relationship between cessation of further exposure to tobacco smoke and the consequent reversible pulmonary changes.

7.4 Langerhans dendritic cells and tobacco smoke cutaneous carcinogenesis

The observations that tobacco interacted with pulmonary LDC (Chapter 4) indicated further analysis was required to investigate further the direct effect of tobacco smoke on LDC. The effect of TSC on murine epidermal

skin during cutaneous carcinogenesis was analysed in this part of the study (Chapter 5). Treatment of the mice skin with TSC led to the increase in the number of epidermal LDC, altered their morphology and impaired their function. This was associated with the development of squamous hyperplasia, dysplasia and consequently the development of skin papilloma and squamous cell carcinoma tumours in all treated mice.

This parallels the previous observations (Chapter 4) where tobacco smoke inhalation increased the density of pulmonary LDC and assumedly impaired their function and consequently this led to the development of the previously described pulmonary lesions e.g. pulmonary Langerhans granulomatosis , bronchial and alveogenic tumours.

The functionally impaired LDC which was induced by tobacco smoke or its condensate in murine lung and skin most likely compromised the anti-tumour immunosurveillance role of LDC and consequently led to the development of the described lesions in lung and skin of the treated mice. This may be related to the suppression of the cellular cutaneous immunity induced by the carcinogen induced prostaglandin release.

The observations on TSC, epidermal LDC and the development of skin tumours in mice is in agreement with simillar observations reported by Muller et al., 1985; Ruby et al., 1989; Andrews et al., 1991, investigating the cutaneous carcinogenesis induced by the tobacco derived carcinogen BP. The suppression of the cellular cutaneous immunity induced by the carcinogen BP induced prostaglandin release was demonstrated elegantly by Andrews et al., (1991) when they implanted prostaglandins synthetase

inhibitor, indomethacin, beneath mice skin before treatment with benzo(a)pyrene and observed delay in the onset of tumour development and reduction in size of the developed skin tumour.

Ten weeks after stopping the TSC treatment, LDC number in skin tumours and in skin around these lesions had not decreased, but significantly increased ($p < 0.0001$). At this stage epidermal LDC became fully dendritic and this was correlated with lymphocytic infiltration into tumours, tumour necrosis, reduction in tumour size and/or tumour regression. It is concluded that the influx of normal LDC into the skin tumours allowed the development of an immune response and consequently tumour necrosis which was followed by reduction in tumour size and/or tumour regression.

In this study it was clearly demonstrated that squamous cell carcinoma of the skin contains a high number of LDC. This parallels the previously reported observations in human well and moderately differentiated squamous cell carcinoma of human lung (Chapter 3) and supported other workers reporting similar findings in human squamous cell carcinomas of other human organs e.g. skin, cervix and oesophagus (Morris et al., 1983; Caorsi and Figueroa, 1984, 1986; McArdle and Muller, 1986; Bonilla-Musoles et al., 1987; Korenberg et al., 1988; Xie, 1990; Matsuda, et al., 1990).

Epidermal LDC recognised TAA (Kato et al., 1977; 1979; Yagi et al., 1987) expressed by the squamous cell carcinoma leading to the increase in the number of fully dendritic epidermal LDC in and around these lesions. The TAA processed by the fully dendritic epidermal LDC, resulted in T-

lymphocyte activation, tumour necrosis, reduction in tumour size and/or tumour regression. These changes presumably reflect an effective immunological anti-tumour response initiated by the expression of TAA in these tumours. Other workers have proposed that tumours may release factors which are chemotactic for Langerhans dendritic cells, hence drawing them into the tumour environment (Halliday, et al., 1992). Cytokines among other chemotactic factors may represent additional factors needed to stimulate the antigen processing and T lymphocytes stimulation by LDC.

7. 5 Precursor of Langerhans dendritic cells

$S100^+$ LDC were identified in normal bone marrow, spleen and in lymph nodes. Their size and morphology varied within these tissues between small, intermediate and large, with or without obvious dendrites on their surfaces. The variation in size and morphology of LDC in tissues is consistent with the previous observations on the $S100^+$ LDC in various lung tumours (Chapter 3) and in agreement with similar observations reported by both Watanabe et al., (1983) and Uccini et al., (1987).

The $S100^+$ cells in the blood and the isolated metrizamide enriched LDC were always small and did not have obvious dendrites extending from their cell membrane. These cells were significantly larger than lymphocytes ($p<0.001$) but smaller than monocytes. The findings in this study support the observations of Watanabe et al., (1983) and Uccini et al., (1987) as they reported the small $S100^+$ lymphocyte-like cells in the blood, lymph nodes and spleen and proposed a lineage relationship between the

S100$^+$ LDC found the these tissues and the small S100$^+$ cells observed in the blood.

When the ultrastructure of the S100$^+$ cells in the blood was examined (Fig. 6.6a&b), the results clearly supported the concept that these blood S100$^+$ cells are in fact related to the S100$^+$ LDC, but different from lymphocytes and monocytes. S100$^+$ cells in the blood had a few short dendrites, S100$^+$ protein granules in the cytoplasm and around the indented nuclear membrane. Their cytoplasm contained numerous and large mitochondria, tubular cytoplasmic structures, single or multivesicular bodies, but no classical BG were seen. This description is totally consistent with the ultrastructure of LDC previously described (Steinman and Cohn, 1973; Van-Voorhis et al., 1982; Dezutter-Dambuyant, 1988; Romani and Schuler, 1989; Steinman, 1991). The cytoplasm of these S100$^+$ LDC did not contain the azurophilic granules found in monocytes (Zucker-Franklin, 1975), and was not granular as in lymphocytes and granulocytes (Zicca et al., 1981; Monahan et al. 1981).

Based on these observations it is proposed that these S100$^+$ cells in the blood represent the precursor of the S100$^+$ LDC found in human bone marrow, spleen and lymphoid tissue.

It is most likely that the S100$^+$ lymphocytes described in the blood by Takahashi and co-workers, 1984, 1985, 1987; De Panfilis et al., (1988); Sansoni et al., (1987); Ferari et al., (1988) and Hanson et al., 1991 were in fact the blood S100$^+$ LDC. This is supported by the observations of other workers investigated the S100$^+$ LDC and other lymphoid cells in various

human tissues (Timens, et al., 1988; Temenia et al., 1990; Gallo et al., 1991; Huang, 1990; Giannini et al., 1991; Cribier, 1992; Shin and Co-workers, 1992, Yamaguchi et al., 1992; Gaillard et al., 1993; Colasante et al., 1993; Paschke et al., 1993; Chaux's group, 1993; Hart and Co-workers, 1993). There has not been a single report describing T-lymphocytes coexpressing S100 protein in their cytoplasm .

Based on the evidence presented in this study, it is proposed that the small $S100^+$ cells found in the peripheral blood, represent the precursors of LDC found in human skin, bone marrow, spleen, lymph nodes and some pathological conditions such as tumours. While they co-express the common leukocyte surface marker CD45 and occasionally weak T cell surface markers, there is no obvious lineage relationship with either T-lymphocytes or monocytes.

7.6 General conclusion

This thesis demonstrated clearly that the density of the antigen presenting Langerhans dendritic cells in tobacco smoke induced human lung cancer is related to tumour type and differentiation. Their highest density in bronchioalveolar carcinoma, well and moderately differentiated SCC was associated with marked TIL in and around these tumours. This was associated with tumour necrosis and longer patients survival.

Why bronchioalveolar, well and moderately differentiated squamous cell carcinomas attracted more LDC than SCLC and poorly differentiated

squamous cell carcinoma is an important question yet to be answered. The relationship between LDC and TAA in certain human tumours deserves further investigation.

It was also demonstrated that tobacco smoke and/or its condensate increased the number of LDC in murine lungs and skin. This was associated with the development of pulmonary Langerhans dendritic cell granulomatosis and other tobacco smoke induced bronchial and alveolar lesions. The increase in number of morphologically altered and functionally compromised LDC induced by TS was associated with the development of pulmonary Langerhans cell granulomatosis, lung and skin tumours. This experiment demonstrated a clear relationship between tobacco smoke exposure and the development of pulmonary Langerhans cell granulomatosis, bronchial and alveolar lesions. This is the first experimental observation of this relationship and implicates pulmonary Langerhans dendritic cells in the pathogenesis of these lesions.

The role of these antigen presenting LDC during tumour development warrants further investigation with special emphasis on their functional status.

While stopping tobacco smoke inhalation was associated with decrease in pulmonary LDC number, their number increased significantly in murine SCC of the skin after ceasing further treatment with TSC. The decrease in their number in murine lungs was associated with reversible changes of all tobacco smoke induced pulmonary lesions with the exception of

bronchial squamous metaplasia which persisted. The continuous increase in their number in skin SCC after stopping further TSC treatment was associated with marked TIL causing tumour necrosis, spontaneous regression of these tumours and/or decrease in tumours size.

It is concluded that tobacco smoke and its condensate increased the number of the functionally compromised LDC. This decreased the cellular immunity and resulted in the development of pulmonary and skin lesions. This implicates the tobacco smoke induced LDC changes in the pathogenesis of tobacco smoke induced lesions.

The increase in their number in well, moderately differentiated SCC of human lungs and in murine SCC of the skin and the associations with longer survival in human and spontaneous regression of murine SCC of the skin indicates an effective anti-tumour response.

The ani-tumour activity of tumour infiltrating LDC, their interaction with tumour cells and TIL is an excellent direction for future research in the field of tumour immunology.

Based on the evidence presented in this study, it is concluded that the small $S100^+$ cells found in the peripheral blood, most likely represent the precursors of LDC found in skin, lymphoid tissue and some pathological conditions such as tumours. While they co-express the common leukocyte surface marker CD45 and weak T cells markers, there is no obvious lineage relationship with either T-lymphocytes or monocytes.

The diagnostic and prognostic values of $S100^+$ LDC in the peripheral blood of patients with malignant diseases, various types of atopic allergy, autoimmune diseases and in patients had organ transplant are yet to be investigated. The pharmacological control of the function of LDC in such conditions warrents study as it may provide an approach to control these conditions.

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S100 POSITIVE DENDRITIC CELLS IN HUMAN LUNG TUMORS ASSOCIATED WITH CELL DIFFERENTIATION AND ENHANCED SURVIVAL

NAIEM A. ZEID AND H. KONRAD MULLER

Department of Pathology, University of Tasmania, Hobart

Summary

Antigen presenting S100 positive dendritic cells have been quantified in normal trachea, lung, bronchial lymph nodes, 130 lung tumors and in 100 lymph nodes regional to tumor. Dendritic cells are rarely seen as intraepithelial components of the normal bronchial mucosa, but are more commonly observed in the perivascular lymphoid tissue of the submucosa and in the alveolar septae of normal lung parenchyma (6 ± 4.85 cells/HPF). The density of these dendritic cells is marked in histologically normal bronchial lymph nodes. Bronchioalveolar (Alveolar II), well and moderately differentiated squamous cell carcinomas contain the highest density of S100 positive dendritic cells, while small cell lung cancer and poorly differentiated squamous cell carcinoma show the lowest density. Regional lymph nodes to lung tumors with lymphocytic predominance and active germinal centres show the highest density of dendritic cells, while unstimulated lymph nodes contain the lowest number of S100 positive dendritic cells. Tumor infiltrating lymphocytes are marked in and around lung tumors with the higher density of dendritic cells. Survival of patients whose tumors contain high density of S100 positive dendritic cells is more favourable compared to tumors with low density of these cells. It is concluded that the density of the antigen presenting S100 dendritic cells in lung tumors is related to subtype, and tumor differentiation. A high dendritic cell density is associated with enhanced patient survival.

Key words: Langerhans cells, S100 positive dendritic cells, human lung tumors, enhanced survival and S100 dendritic cells.

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INTRODUCTION

Langerhans cells (LC) were the first dendritic cell to be described in the epidermis. The unique ultrastructural marker of this cell is the cytoplasmic organelle, the Birbeck granule. The bone marrow origin of LC was first reported by Frelinger et al.[1] and demonstrated by the elegant experiments of Katz and colleagues.[2]

LC are widely distributed in human skin, esophagus, cervix, ocular and buccal epithelia.[3] Other related dendritic cells have been reported in the dermis of the skin (indeterminate dendritic cells), in the afferent lymph (veiled dendritic cells), in spleen and lymph nodes (interdigitating dendritic cells) and interstitial dendritic cells in the connective tissue of all organs but the brain.[4] Little is known about the precursor of LC in the bone marrow.

The primary function of LC and other related dendritic cells is to process and present antigen to T lymphocytes in the regional lymph nodes to initiate an immune response.[5,6]

LC were first reported in human tumors (cervix) by Younes et al.[7] Since then LC have been reported in other tumors: bronchioalveolar tumor of lung,[8] laryngeal carcinoma,[9] pleomorphic adenoma of salivary gland,[10] skin tumors,[11,12] lung,[8,21,22] breast and thymoma,[3] esophageal[13] and gastric tumors,[14] papillary thyroid carcinoma,[15] colorectal carcinoma,[16] nasopharyngeal carcinoma,[28] and prostatic carcinoma.[29] S100 protein was used as a marker for LC in most of the above reports. Hammar et al.[3] reported Birbeck granules in all the S100 positive dendritic cells in lung bronchioalveolar cell carcinoma and adenocarcinoma, nasopharyngeal, ovarian, breast carcinomas, thymoma and in malignant histiocytosis.

Basset and colleagues[8] first reported dendritic cells in the bronchio-alveolar lavage fluid of patients with primary alveolar carcinoma of the lung similar in ultrastructural features to LC. Since then Kawanami et al.[17] and Richard et al.[18] have described LC with characteristic features in normal human lung. Sertl et al.[19] and Holt and Schon-Hograd,[20] have also reported Ia positive dendritic cells in the bronchial epithelium and in the alveolar septum of normal lungs of mice, humans and rats.

In human lung tumors there are conflicting reports on dendritic cells and prognosis. While Furukawa et al.[21] associated longer survival with marked infiltration of LC in stage 1a adenocarcinomas of the lung, Fox et al.[22] suggested that a high number of LC in lung tumors is associated with a worse prognosis. They also reported CD1 positive LC mainly in squamous cell carcinoma and adenocarcinoma of the lung.

In the present study S100 positive dendritic cells have been quantified in normal trachea, lung, 130 lung tumors and in regional lymph nodes draining lung tumors.

MATERIALS AND METHODS

Specimens

Paraffin blocks of 130 lung tumors, 100 regional lymph nodes, 13 normal lung and 7 normal bronchial lymph nodes were obtained from the files of the Department of Anatomical Pathology, Royal Hobart Hospital. Three 3-4 micron sections were cut from each block, one for S100 staining, a second for antibody control and a third for hematoxylin and eosin staining.

Clinical data

All data related to patients involved in this study were obtained from the files of the Medical Record Department, Royal Hobart Hospital.

Preparation of tracheal and bronchial mucosal sheets

Four segments, 2 × 1 cm each of unfixed trachea and major bronchus from 2 autopsies were incubated in 20 mm solution of EDTA, pH 7.2 (ethylene diamine tetraacetic acid-Na4-salt, Serva 11282). The incubation time and temperature were modifications of those suggested by Scaletta and MacCallum.[23] The mucosal segments were incubated for 3 hrs at 37 °C and overnight at 4 °C. The tracheal and bronchial segments were treated with 50 per cent ethanol alcohol for 30 mins. The mucosal sheets were separated gently from the underlying cartilage, flattened on filter paper and processed for routine paraffin embedding; mucosal segments were blocked in paraffin as a flat mucosal sheet. The whole tracheal and bronchial mucosa were tangentially and serially sectioned at 3 micron. A total of 159 sections were prepared for immunohistology and routine staining.

Immunohistology

Rabbit antibody to bovine S100 protein (Dakopatts, Denmark, Z 311) was used to identify the dendritic cells in paraffin sections. The paraffin wax of sections was first melted at 56 °C for 2 hrs, dried overnight at 37 °C and then the paraffin wax remelted at 56 °C for 5 mins before dewaxing in 2 changes of xylene, 5 mins each. Sections were hydrated in descending concentration of ethanol alcohol to water. Dewaxed sections were placed in pre-warmed phosphate buffer saline (PBS), pH 7.4 at 37 °C for 5 mins. Enzyme digestion was then carried out by placing the sections in pre-warmed 360 mL PBS, pH 7.4, containing 0.02 gm of protease (Sigma No. 8038) and incubated at 37 °C for 6 mins. After washing the sections in running water for 5 mins they were rinsed in 0.05 M TRIS-HCL saline, pH 7.6, for 5 mins. After drying, S100 antibody diluted 1:400 in diluent (1% bovine serum albumin, Sigma A 7030 and human serum group AB, CSL 1121001) was applied to the sections and incubated for 90 mins at room temperature. Sections were washed in 0.05 TRIS-HCL, pH 7.6, for 5 mins. Sections were then incubated for 50 mins at room temperature with the secondary antibody (biotinylated anti-rabbit immunoglobulin, Biogenex HK 326-UR) diluted 1:500 as for S100 antibody. After washing sections in 0.05 M TRIS-HCL saline, they were incubated with label streptavidine alkaline phosphatase (Biogenex UK 321 UK) diluted 1:50 in TRIS-HCL 0.05 M pH 7.6 and incubated at room temperature for 50 mins. Sections were rinsed in 0.1 M TRIS-HCL pH 8.2 after washing in 0.05 M TRIS-HCL pH 7.6. The substrate (naphthol phosphate in Tris buffer) and the chromagen, Fast Red (fast red TR salt and levamisole, Biogenex, USA, HK 182-5K) were applied to the sections and incubated at room temperature for 20 mins and then counterstained in Mayers hematoxylin for 3 mins after washing in de-ionised water for 5 mins. Following washing in de-ionized water sections were mounted in crystal/mount (Biomeda, USA, M02), cleaned in xylene and finally mounted in Eukitt (Carl Zeiss, West Germany, 00 20 02).

Identification and counting of S100 positive dendritic cells

The S100 positive dendritic cells were identified by their red cytoplasm, indented nuclear membrane and prominent nucleolus (Fig. 1). Chondrocytes of bronchial cartilage, myoepithelial cells of the serous glands in the lamina propria and nerve fibres are S100 positive in lung sections, but these cells were readily distinguished from the S100 positive dendritic cells.

S100 positive dendritic cells (DC) were counted in 4 high power fields (× 400) for each section. These fields covered the tumor itself and zonal junction between the tumor and the surrounding lung parenchyma. The mean of the dendritic cells number in the 4 high power fields and the standard deviation were calculated for each case.

The relationship between the S100 positive DC in each type of lung tumor and the density of tumor infiltrating lymphocytes (TIL) in and around tumors was recorded. The density of TIL have been evaluated as mild, moderate and marked.

Counting the S100 positive DC in lymph nodes was done following the same methodology applied to lung sections.

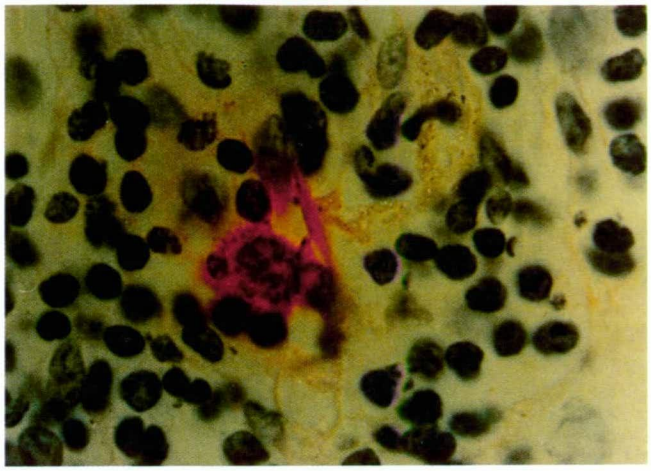

Fig. 1 S100 positive dendritic cell (DC) shows red cytoplasm and indented nucleus (Immunoperoxidase Technique × 250).

RESULTS

Normal tracheal, bronchial mucosa and lung

S100 positive DC were rarely found in the intraepithelial component of the bronchial tree (Fig. 2). They were commonly observed in the lamina propria of the tracheal and bronchial mucosa close to the perivascular lymphoid tissue. An average of 2 S100 positive DC were present in both tracheal and bronchial sections in every 3 HPF.

DC were observed in the alveolar septum of all sections of normal lung, but in various densities. Occasionally DC were found in the alveolar spaces (Fig. 3). The mean of S100 positive DC count in 4 high power fields was 6 ± 4.85 cells in normal lung.

Dendritic cells in lung tumors

S100 positive DC were found in direct contact with TIL and tumor cells in all varieties of lung tumors, but with various densities. Bronchioalveolar (Fig. 4) and well and moderately differentiated squamous cell carcinomas (Fig. 5) showed the highest number of these cells within tumors. Small cell lung cancer, mucus producing

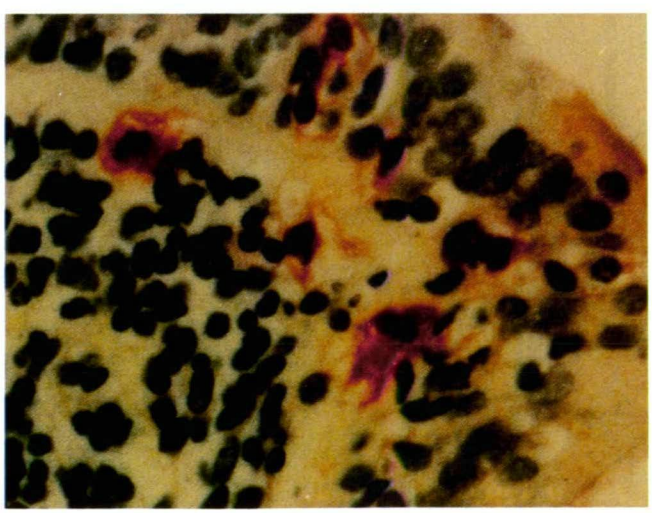

Fig. 2 S100 positive DC in the bronchial epithelium and in the bronchial lymphoid tissue (Immunoperoxidase Technique × 400).

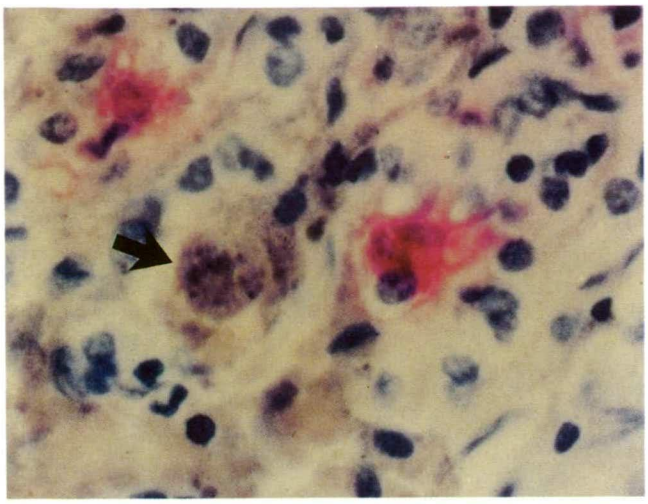

Fig. 3 S100 positive DC in the alveolar spaces. Alveolar macrophages (arrow) are negative for S100 protein (Immunoperoxidase Technique ×400).

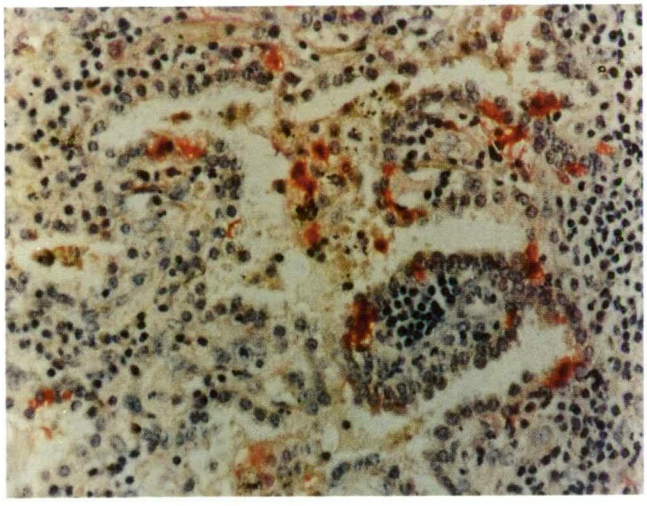

Fig. 4 S100 positive DC in bronchioalveolar carcinoma (Immunoperoxidase Technique ×250).

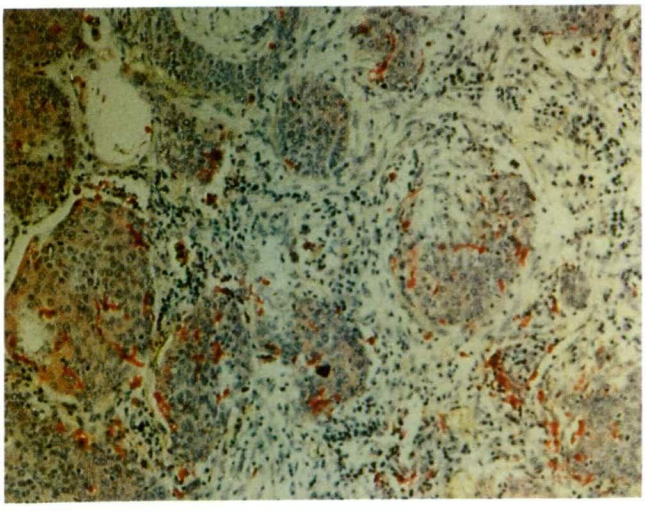

Fig. 5 S100 positive DC in well differentiated squamous cell carcinoma (Immunoperoxidase Technique ×100).

adenocarcinomas and poorly differentiated squamous cell carcinomas contained the lowest number of S100 positive DC. Table 1 summarizes these results.

Dendritic cells morphology in lung tumors

The number of DC dendrites in relation to the histological variety of the lung tumors were examined. DC were divided into 2 groups according to the number of dendrites extending from the cell body, first DC with more than 3 dendrites and secondly DC with less than 3 dendrites. DC with more than 3 dendrites were found in 77.8% of bronchioalveolar carcinomas, 67.4% of well and moderately differentiated squamous cell carcinomas, 34.7% of all varieties of adenocarcinomas and in 32.6% of poorly differentiated squamous cell carcinomas. Dendritic cells with less than 3 dendrites were more commonly seen in the majority of small cell lung cancer and poorly differentiated squamous cell carcinoma.

Contact between DC and lung tumor cells

Direct contact between S100 positive DC and tumor cells (Fig. 6) was consistently present in all bronchioalveolar (alveolar II) carcinomas, in all well and moderately differentiated squamous cell carcinomas and in 81.3% of all varieties of adenocarcinomas. Contact between DC and tumor cells was rarely observed in poorly differentiated squamous cell carcinomas and small cell lung cancers, although LC were present in these lesions.

Contact between DC and TIL in lung tumors

Direct contact between DC and TIL were found in all varieties of lung tumors (Fig. 7). In general there was a proportional relationship between the number of DC and the density of TIL in and around various lung tumors. The density of TIL was marked in bronchioalveolar, and in well and moderately differentiated squamous cell carcinomas. The density of TIL was moderate in other varieties of adenocarcinomas and mild in both poorly differentiated squamous cell and small cell lung cancer.

Central tumor necrosis, DC number and TIL density in lung tumors

Central tumor necrosis was evident in lung tumors with high number of DC and marked density of TIL. Marked

TABLE 1 S100 positive DC in lung tumors

Histology	No. of cases	Mean of S100 + DC	SD
1. Bronchioalveolar	16	57.8	26.5
2. Well differentiated squamous cell carcinoma	16	42.6	21.2
3. Moderately differentiated squamous cell carcinoma	14	37.4	10.8
4. Poorly differentiated squamous cell carcinoma	38	13.2	10.8
5. Nonciliated and Clara cell adenocarcinomas	18	25.06	11.4
6. Ciliated and mucous producing adenocarcinomas	14	12.35	5.7
7. Adenosquamous carcinoma	4	24.25	8.2
8. Small cell lung cancer	10	4.7	3.8
Normal lung	13	6	4.85

(Using the non-parametric, Wilcoxon test, 1 verse 4, 5, 6, and 8 was significantly different, $p<0.0005$). (2 verse 4, 5, 6, and 8 also significantly different, $p<0.0001$, 0.0125, 0.0001 and 0.0003 respectively).

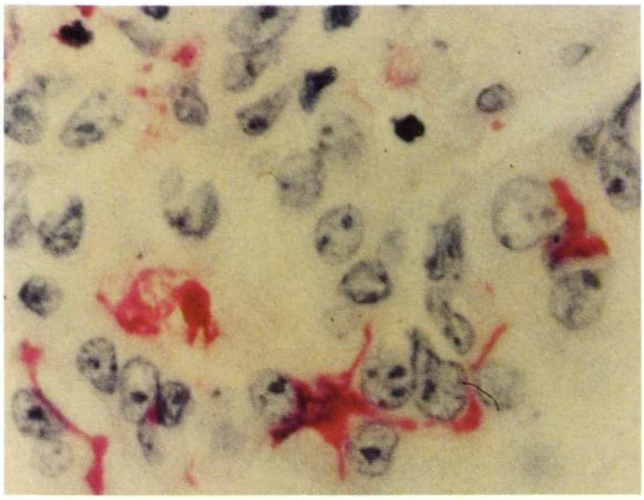

Fig. 6 Direct contact between S100 positive DC and tumor cells, adenocarcinoma (Immunoperoxidase Technique ×100).

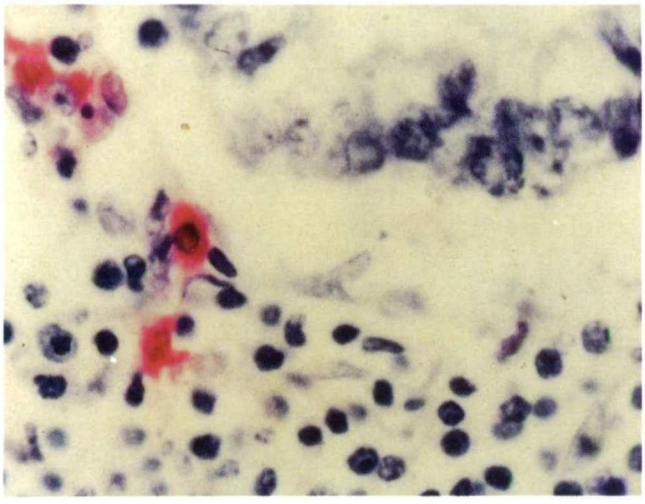

Fig. 7 Contact between S100 positive DC and tumor infiltrating lymphocytes (TIL) (Immunoperoxidase Technique ×400).

central tumor necrosis was found in 70% of well and moderately differentiated squamous cell carcinomas as well as in 30% of the small number of cases with bronchioalveolar carcinomas.

Dendritic cells in lymph nodes regional to lung tumors

Based on the histological evidences of immunological activity,[24] lymph nodes regional to the lung tumors examined in this study have been divided into 3 groups. Firstly lymph nodes with lymphocytic predominance, secondly lymph nodes with active germinal centres and thirdly an unstimulated group of lymph nodes. S100 positive DC were found in the paracortical T lymphocyte area (Fig. 8). The density of S100 positive DC in various groups of lymph nodes are shown in Table 2. The density of S100 positive DC was high in lymph nodes showing lymphocytic predominance and in lymph nodes with active germinal centres. The unstimulated lymph nodes showed the lowest density of DC. The density of the S100 positive DC in bronchial lymph nodes regional to normal lung was higher than unstimulated lymph nodes.

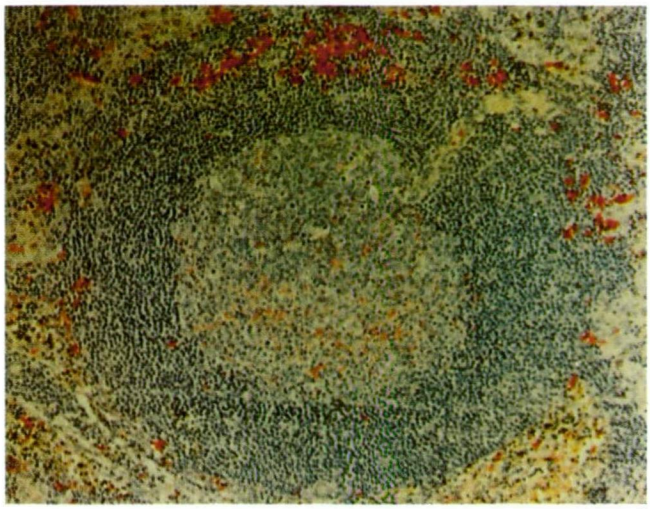

Fig. 8 S100 positive DC in the T-zone of lymph nodes (Immunoperoxidase Technique ×250).

The dendritic nature of DC was recorded in all varieties of lymph nodes examined. About 93% of DC in lymph nodes with lymphocytic predominance show more than 3 dendrites, compared to 89% in lymph nodes with active germinal centres and to less than 50% in unstimulated lymph nodes. The number of dendrites reflected the state of immunological activity of these lymph nodes.

S100 positive DC and patients' survival with lung cancer

When patients were divided into those with the highest number of S100 positive DC in their tumors and a second group with the lowest number of DC (see Table 3), important comparisons were evident.

The density of TIL was marked in tumors with the highest number of DC and it was scanty in tumors with the lowest number of DC. The overall survival of patients in the former group was almost twice that of the second group. About 92.6% of the cases in the better surviving group are well, moderately differentiated squamous cell and bronchioalveolar lung carcinomas, while small cell lung cancer, poorly differentiated squamous cell and other varieties of adenocarcinomas formed about 85.2 per cent of cases in the second group.

The M:F ratio in the 2 groups was 3:1. Patients in both groups smoked an average of 28 cigarettes a day for some 37 yrs. There was no difference in tumor size or in the incidence of lymph node metastasis in both groups.

TABLE 2 S100 positive DC in regional lymph nodes

Histology of lymph nodes	No.	Mean of S100 + DC	SD
Lymphocytic predominance (A)	27	69.26	28.88
Active germinal centre (A)	46	69.67	28.42
With metastasis (A)	16	69.95	43.92
Unstimulated (B)	33	24.82	13.43
Normal lymph nodes (B)	7	35	10.23

Based on the Bonferroni multiple-comparison test, lymph nodes with (A) were not significantly different at the 5% level, while groups (A) and (B) were significantly different.

TABLE 3 Lung tumors with the highest and lowest number of S100 positive DC

	Highest No. of DC	Lowest No. of DC
1. No. of cases	27	41
2. S100+ DC/HPF	69.8 ± 19.40	6.55 ± 3.53
3. Density of TIL	Marked	Scanty
4. Average tumor size (cm)	3.53 ± 1.43	4.7 ± 3.26
5. LN metastasis	33.3%	34.3%
6. M:F ratio	3:1	3:1
7. Cigarettes/day	28.69 ± 15.39	27.62 ± 17.18
8. Smoking years	37.23 ± 10.32	36.27 ± 13.3
9. No. of dead	8 (29.6%)	28 (68.6%)
10. Survival (months) since diagnosis	68.46 ± 32.87	8.3 ± 7.72
11. Histological typing	D.SCC 59.3% BA 33.3% ADENO 7.4%	PDSCC 55.17% ADENO 31.03% SCLC 13.79%

D.SCC = well and moderately differentiated squamous cell carcinoma. PDSCC = poorly differentiated squamous cell carcinoma. BA = bronchioalveolar carcinoma. ADENO = adenocarcinoma. SCLC = small cell lung carcinoma.

DISCUSSION

This study demonstrates that in lung tumors the density of antigen presenting S100 positive dendritic cells varies according to cellular subtype and differentiation of lung carcinomas. Bronchioalveolar (alveolar II), well and moderately differentiated squamous cell carcinomas contain the highest number of dendritic cells. The density of TIL is in direct proportional relationship with the number of S100 positive dendritic cells. Patients with high number of dendritic cells in their lung tumors, e.g. bronchioalveolar, well and moderately differentiated squamous cell carcinomas, survived longer than patients with a low number of dendritic cells, e.g. poorly differentiated squamous and small cell lung cancer, i.e. 68.46 ± 32.87 mths compared to 8.3 ± 7.72 mths.

S100 positive DC with more than 3 dendrites were the majority in bronchioalveolar, well, and moderately differentiated squamous cell carcinomas of the lung and in lymph nodes with lymphocytic predominance and active germinal centres. These findings favour the hypothesis that the increase in number of LC dendrites in certain tumors may reflect an active immunological state.

Contact between S100 positive DC, tumor cells and T lymphocytes were investigated in the varieties of lung tumors and in lymph nodes. Direct contact between these antigen presenting cells and tumor cells was observed in all bronchioalveolar, well differentiated, moderately differentiated carcinomas and in the majority of adenocarcinomas. Direct contact was also observed between T lymphocytes and DC in lung tumors and in the T-zone of lymph nodes. This contact may reflect the immune interaction between DC, tumor cells and T lymphocytes. This has also been reported in human skin tumors.[11]

Central tumor necrosis in the investigated lung neoplasms was related to high density of S100 positive DC and marked infiltration of TIL. This was observed in well, moderately differentiated squamous cell and in bronchioalveolar carcinomas. This may result from the effect of sensitized T lymphocytes acting specifically against the target tumor cells.[4,6]

The current study shows that the density of dendritic cells in lymph nodes regional to lung tumors is directly related to the immunohistological activity of these lymph nodes. Lymph nodes showing active germinal centres or lymphocytic predominance contained higher numbers of S100 positive dendritic cells than the unstimulated nodes.[24]

Kitaichi et al.[24] reported that the 3 yr survival of patients in stage 2 and 3 of lung cancer in which the regional lymph nodes showed lymphocytic predominance or active germinal centres was better than in patients with unstimulated lymph nodes.

Furukawa et al.[21] also reported that patients with a marked number of S100 positive DC in and around their lung adenocarcinomas survived longer than patients without or with minimal infiltration of these cells. Temeck et al.[25] found 65.25% of patients with carcinomas of the lung alive 10 yrs after diagnosis and treatment were epidermoid carcinomas. Patients with adenocarcinoma represented 33.05%, while there was only one patient with small cell lung cancer and one patient with large cell lung cancer alive. A similar study by Kern et al.[26] reported 46.36% of patients with carcinomas of the lung who survived an average of 15.5 yrs after diagnosis and treatment had epidermoid carcinomas, 25.45% adenocarcinomas and 11.81% bronchioalveolar carcinomas. In a recent study of 9090 patients with carcinomas of the lung, Watkin et al.[27] reported a longer survival for squamous cell carcinomas than small cell lung cancer. A relationship between the increased density of dendritic cells in other human tumors and longer patient survival has been reported during the last 5 yrs, e.g. nasopharyngeal tumors,[28] gastric tumors,[14] papillary thyroid carcinomas,[15] colorectal adenocarcinomas[16] and squamous cell carcinomas of the esophagus.[13]

This study also demonstrated that S100 positive dendritic cells are present in normal trachea and bronchus, normal lung parenchyma and normal bronchial lymph nodes. These dendritic cells were more commonly observed in the alveolar septum of normal lung but occasionally were seen in the normal tracheal or bronchial mucosa. These observations are in agreement with Sertl et al.,[19] Richard et al.[18] and Holt and Schon-Hegrad.[20]

The primary function of dendritic cells is the presentation of antigen to T lymphocytes in the regional lymph nodes.[5,6] The selective increase in the number of DC in bronchioalveolar, well and moderately differentiated squamous cell carcinomas may be related to specific tumor associated antigen.[4,19] Alternatively, tumors may release factors which are chemotactic for dendritic cells, hence drawing them into the tumor environment.[30]

In conclusion this study has clearly demonstrated that the density of S100 positive DC in lung tumors was related to cellular subtype and differentiation of tumor cells. Bronchioalveolar, well and moderately differentiated squamous cell carcinomas attracted the highest number of DC, and this was associated with marked density of TIL and better survival. The mechanism by which the increased number of antigen presenting cells accumulate in tumors warrants detailed investigation.

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Address for correspondence: H.K.M., Department of Pathology, University of Tasmania Clinical School, 43 Collins Street, Hobart, Tas 7000.

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TOBACCO SMOKE INDUCED LUNG GRANULOMAS AND TUMORS: ASSOCIATION WITH PULMONARY LANGERHANS CELLS

NAIEM A. ZEID AND H. KONRAD MULLER
Department of Pathology, University of Tasmania, Hobart

Summary

The density of zinc-iodide-osmium (ZIO) positive pulmonary Langerhans dendritic cells (LC) was increased about 20-fold in mice after passive exposure to tobacco smoke. This was associated with pulmonary changes consistent with the cigarette smoking-related clinical syndrome in humans, pulmonary Langerhans cell granulomatosis. The major feature was an interstitial peribronchial granuloma. The cellular infiltrate of the granuloma (lymphocytes, plasma cells, eosinophils, clusters of large histiocyte-like cells and macrophages) extended into the adjacent alveolar septum forming a star-shaped lesion. The histiocyte-like cells were large with pale acidophilic cytoplasm and many ill-defined short dendrites extending from the cell membrane. Bronchial epithelial metaplasia also developed. The interstitial changes were followed by the development of proliferative alveolar and bronchial lesions in 2 mice. The zinc-iodide-osmium positive cells were consistent with Ia positive pulmonary dendritic cells and their ultrastructure was similar to that of pulmonary Langerhans cells. After ceasing exposure to tobacco smoke the density of pulmonary Langerhans cells returned to that of the control level; interstitial granulomatous lesions disappeared, but the bronchial epithelial metaplasia did not reverse. Tobacco smoke exposure of mice produces interstitial granulomatous inflammation similar to Langerhans cell granulomatosis in humans. The elevated level of pulmonary Langerhans cells implicate these cells in the pathogenesis of these lesions.

Key words: Langerhans cell, tobacco smoke, pulmonary histiocytosis X, Langerhans cell granulomatosis.

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INTRODUCTION

Histopathological changes in the lungs induced by cigarette smoking have been documented in humans,[1,2] and experimental animals.[3,4] Auerbach et al.,[2] reported tobacco smoke induced histologic changes in human tracheobronchial epithelium. These included basal cell hyperplasia, stratification and squamous cell metaplasia of the bronchial epithelium. The epithelial changes were more frequent in patients with lung cancer than in cancer-free smokers.

The carcinogenic effects of tobacco smoke on the lungs of laboratory animals have been investigated by many workers.[3-7] These studies have concentrated on tobacco smoke induced precancerous changes and tumor development. In mice, the pulmonary changes induced by tobacco smoke include bronchitis, atypical bronchial epithelial proliferation and occasional adenomatous lung tumors.[3]

Casolaro,[8] reported an increase in the number of CD1 positive dendritic cells in the bronchio-alveolar lavage fluid of smokers, while Soler et al.,[9] described an increase in both $T6^+$ LC and $M241^+$ dendritic cells in smokers' lung. A relationship between tobacco smoke inhalation and pulmonary interstitial granulomas has been reported in humans — 90% of patients with pulmonary histiocytosis X were smokers.[10-13]

Basset and Turiaf,[14] together with Nezelof et al.,[15] established that the pulmonary histiocytosis X cell is a Langerhans cell. These cells were isolated from bronchio-alveolar lavage fluid and characterized ultrastructurally. An excellent review of LC granulomatosis (histiocytosis X) was published by Hance et al.[10] The 3 clinical syndromes, Letterer-Siwe disease, Hand-Schüller-Christian syndrome and eosinophilic granuloma were grouped together and called histiocytosis X, based on the similarities of the lesions.[16,17] Pulmonary Langerhans cell granulomatosis (pulmonary histiocytosis X) is slightly different from the other varieties of histiocytosis X. The former is commonly seen in young male adults,[13] but not in children as are the other forms of histiocytosis X.

Katzenstein and Askin,[11] described the detailed histologic features of pulmonary eosinophilic granuloma which included bronchiocentric, patchy and nodular lesions due to interstitial infiltration with a mixture of histiocyte-like cells and variable numbers of eosinophils, plasma cells and lymphocytes. The nodular interstitial lesions may appear "star-like" due to cell infiltration and fibrosis along the alveolar septum. A DIP-like reaction (desquamative interstitial pneumonia-DIP), with accumulation of alveolar macrophages in the surrounding alveolar spaces may be seen but is not specific for pulmonary LC granulomatosis. Bronchiolitis is frequently observed and spontaneous healing of pulmonary LC granulomatosis may take place leaving a star-shaped scar.[11]

The diagnostic cell in this lesion is the Langerhans dendritic cell or histiocytosis X cell. It is a large cell with an ill-defined border, abundant eosinophilic cytoplasm, and a folded or indented nucleus with an inconspicuous nucleolus. There are many of these cells in active lesions, but they become rare in older fibrotic lesions.[11] Birbeck granules (BG) in both epidermal LC and histiocytosis X cells show the same affinity for zinc-iodide-osmium (ZIO) staining,[18] both cells are S100,[19] and OKT6 positive.[15] The ultrastructural features of histiocytosis X cells are consistent with those of LC.[18,20] However, the BG in the histiocytosis X cell appear to be more often attached to the cell membrane than those in epidermal LC.

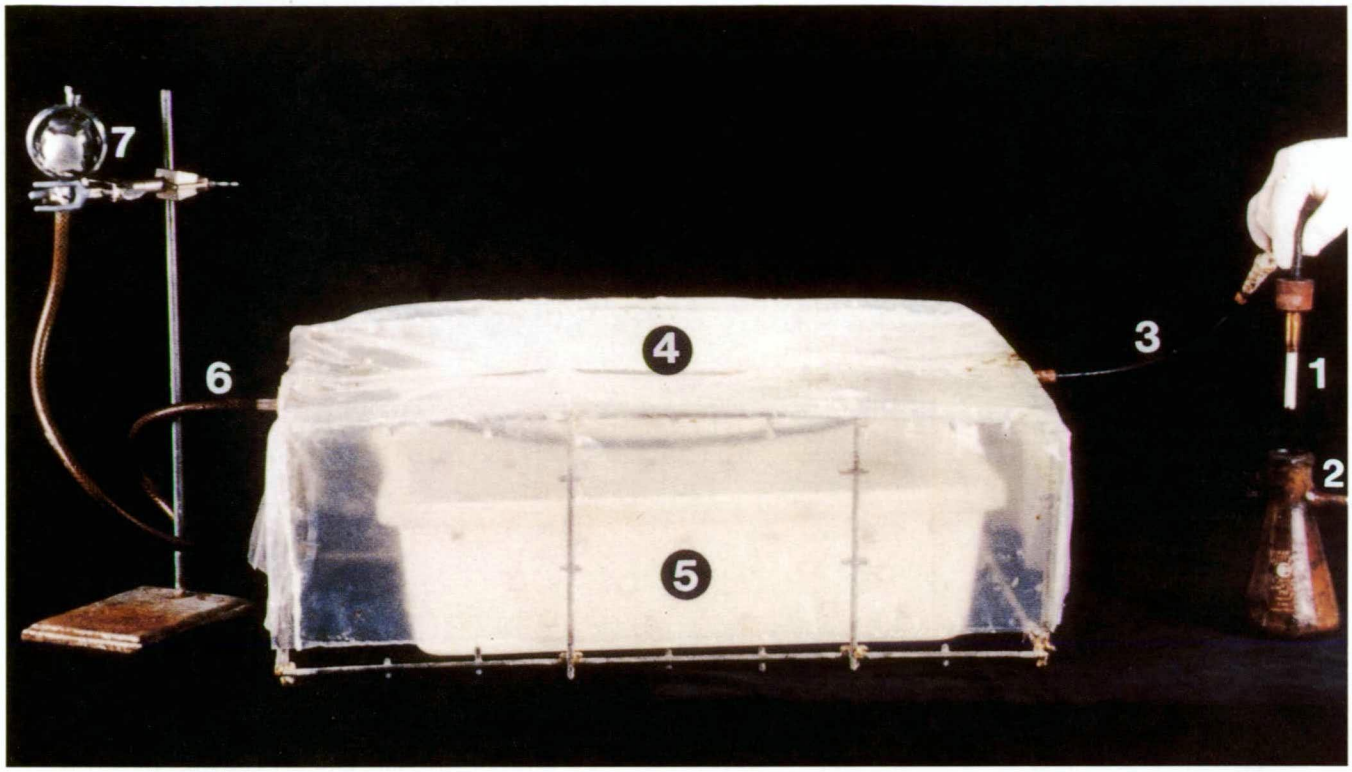

Fig. 1 Tobacco smoke chamber consisting of smoke generating unit with a cigarette holder (1) connected by plastic tube (2) to a central continuous source of air. This was connected by plastic tube (3) to the smoke exposure tent (4) which covered the mouse cage (5). The air outflow unit was composed of a safety smoke outlet (6) and adjustable central suction (7).

The aim of this study was to investigate experimentally the effect of tobacco smoke on pulmonary LC in mice. The associated pulmonary lesions which developed are described.

MATERIALS AND METHODS

Animals

BALB/c female mice 6-8 wks old were obtained from the Central Animal House at the University of Tasmania after Animal Ethics Committee approval. The 108 mice used for the tobacco smoke inhalation experiment were divided into 2 groups — experimental and controls, each of 54 mice. Control mice were kept separately in groups of 12 during the experiment, while the experimental mice were kept in groups of 6 mice.

Experimental design

Experimental mice were exposed to the tobacco smoke of 8 cigarettes every day including weekends. Each cigarette needed 2 mins to burn completely, so the mice were exposed for a maximum of 16 mins each day. The smoke:air ratio used in this study was 1:12; other researchers have used ratios of 1:7.[21-23] An appropriate smoke:air ratio is vital to protect the mice from acute smoke toxicity and death.

During the first week of the experiment, mice were exposed to the smoke of only one cigarette a day to acclimatize them to the much higher dose of 8 cigarettes during the second week. Six test and 6 control mice were killed by cervical dislocation at wks 1 and 2 and then every 4 wks. One lung from each mouse was snap-frozen in liquid nitrogen and stored in a $-80\,^{\circ}C$ freezer for ZIO and anti-Ia immunoperoxidase staining. The other lung together with the trachea and bronchi was perfused overnight in 10% formalin in PBS (pH 7.3), and processed for histological examination. At 28 wks histological examination of the lungs of the test group showed positive tobacco induced changes. The remaining mice ceased to be exposed to further tobacco smoke and were kept for another 6 wks to study the reversibility of the tobacco induced changes in the lung.

Tobacco smoke chamber

The tobacco smoking chamber (Fig. 1) was designed by one of the authors (N. A. Z). It consisted of 3 units, a smoke generating unit, a smoke exposure tent and an air outflow unit.

The smoke generating unit was a 500 mL filter (Buchner flask, Selbys, Cat No 414644, FB500/3) connected by plastic tube to a central continuous source of air. The rate of air flow can be adjusted by a valve switch. A cigarette holder was fitted in the centre of the cover of the flask. The mouthpiece of the cigarette holder was connected by plastic tube to the smoke exposure tent. The central air flow was switched on before lighting the cigarette and firmly closing the flask. The air flow to the flask was adjusted to ensure the 2 mins' time needed for complete burning of the cigarette. The air in the flask together with the generated tobacco smoke flowed through the cigarette holder and plastic tube to the smoking tent. The amount of smoke generated from burning one untipped cigarette was about 350 mL.

The smoke exposure tent was designed to provide the mice in the tent with an environmental air similar to that of any enclosed place in the human situation. It consisted of the plastic tent and the mouse cage. The tent was constructed from transparent plastic and supported by a steel frame. The tent was not sealed at the lower margin in order to provide continuity and similarity between the tent and the room environmental air. The tent was provided with a smoke inlet and safety smoke outlet connected to a central adjustable air suction. A standard animal cage was used provided with 74 holes at all sides, each hole about 1 cm in diameter. The cage measured $50 \times 33 \times 13$ cm.

The air outflow unit consisted of a safety smoke outlet connecting the tent to the adjustable central suction.

Tissue preparation

Representative sections from the lung and bronchial lymph nodes of both control and test groups were processed and vacuum embedded in wax. Four μm sections were cut and stained with Haematoxylin and eosin for light microscopy examination.

ZIO procedure to visualize Langerhans dendritic cells

A modified ZIO procedure,[24,25] was developed because of the nature of the lung. The ideal size and thickness of the lung tissue were 5×5×2 mm to ensure complete penetration of the fixative and staining solution. The frozen lung sections were fixed in V-7.4-ZIO (veronal sodium-HCl buffer solution — pH 7.4-ZIO) at 4 °C for 19 hrs and then processed for routine paraffin embedding after washing in running tap water for 5 mins. Five μm sections were cut, dewaxed in 2 changes of xylene, 5 mins each and mounted directly in Eukitt mounting media (Carl Zeiss, West Germany, 00 20 02).

Preparation of V-7.4-ZIO solution

A mixture of 2.4 gm zinc powder (BDH Australia, Prod No 30596) and 1 gm of iodine bisublimate (Ajax, Sydney-Melbourne, 267, 70516) was added to 40 mL of 0.2 M 7.4 veronal sodium-HCl buffer. After shaking the mixture for 10 mins the solution was immediately filtered. The filtered solution was mixed with 2% osmium tetroxide (OsO_4, BDH Australia, Prod No 29422) in a ratio of 3:1. This solution was prepared fresh for each experiment.

V-7.4-ZIO staining for electron microscopy examination

Modifications of the procedure of Rodriguez and Caorsi,[25] were used. Lung sections 25 μm thick were cut from paraffin blocks of lung tissue which had been already incubated with V-7.4-ZIO solution. They were dehydrated in ascending concentration of ethanol alcohol, 20 mins each and embedded in Epon resin.

Immunoperoxidase staining to visualize Langerhans cells

Five μm acetone-fixed frozen sections of lung tissue were mounted on potassium-dichromate-gelatine coated slides, dried for 2 hrs, incubated with mouse monoclonal anti-Ia (MKD6) for 90 mins at room temperature, washed 3 times in phosphate-buffered saline (PBS, pH 7.4) over 20 mins, and incubated with horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin (HRPO-anti-Ig, 1:80; Dakopatts, Denmark). Sections were washed in PBS 3 times over 20 mins. The Ia positive cells were identified by incubating the sections with 0.5 mg/mL diaminobenzidine (DAB, Sigma) in PBS containing 0.02% H_2O_2, pH 7.4 for 10 mins. Sections were washed in running water over 10 mins and counterstained with Mayers Haematoxylin. The washed sections were mounted in glycerol gelatine (Sigma, 86F-6098) for microscopic examination.

Enumeration of ZIO positive LC in lung sections

The number of ZIO positive LC in 4 high power fields (HPF×400) was calculated using standard fields and the same microscope and objectives throughout the study. The mean and the standard deviation were statistically analyzed for groups of mice assessed.

RESULTS

Pulmonary changes induced by tobacco smoke

Bronchial epithelial hyperplasia, focal alveolar cell hyperplasia, mononuclear and eosinophil infiltration of the alveolar septum, together with alveolar macrophages in the alveolar spaces were observed in lungs of mice after 4 wks of exposure to tobacco smoke. By the end of week 8, multifocal interstitial lung lesions were present

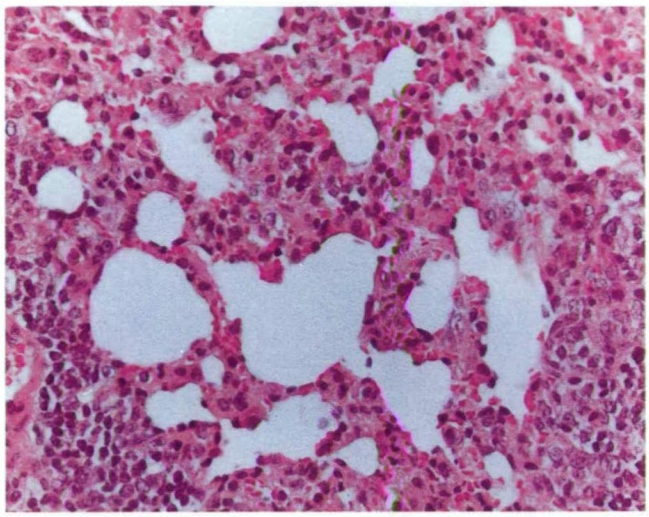

Fig. 2 Increase in thickness of the alveolar septum due to increased numbers of mononuclear cells. (Haematoxylin and eosin, original magnification ×100).

composed of monocytes and lymphocytes (Fig. 2). This was observed in the lungs of 6 mice examined at this time.

After 12 wks of tobacco smoke exposure, interstitial granulomas without central necrosis or cavitation were observed in lungs of 3 of 6 mice. Cholesterol-like crystals (Fig. 3) were present between the cells forming these granulomas as well as histiocyte-like cells, foamy macrophages, lymphocytes, plasma cells and eosinophils. The alveolar spaces around the granulomas showed alveolar epithelial hyperplasia with marked accumulation of macrophages.

By 16 wks, bronchial epithelial hyperplasia and occasional focal bronchial squamous metaplasia were observed (5 of 6 mice). The lung of one mouse at this stage showed a complex of histopathological changes. The basic lesion was peribronchial chronic inflammation (Fig. 4). Some lesions contained granulomas and there was a central cavity (Fig. 5). The cellular infiltrate of the granuloma extended in the adjacent alveolar septum

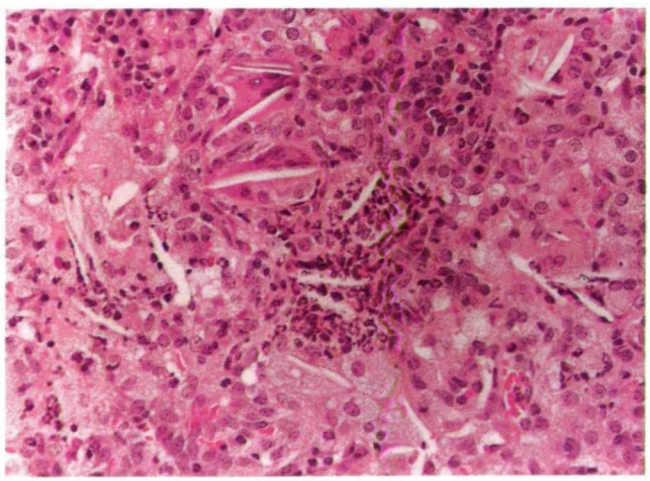

Fig. 3 Interstitial granuloma showing cholesterol crystals, mononuclear cells and foamy alveolar macrophages. (Haematoxylin and eosin, original magnification ×200).

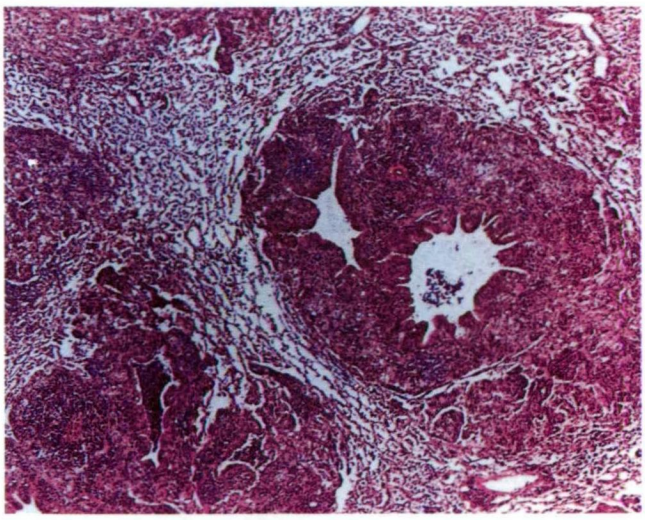

Fig. 4 Multiple peribronchial interstitial granulomatous inflammation. (Haematoxylin and eosin, original magnification ×100).

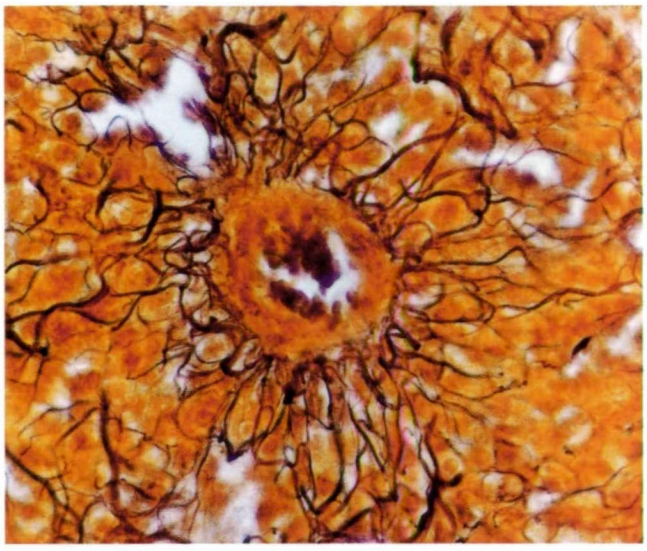

Fig. 6 Star-shaped lesion of the interstitial pulmonary granuloma. (Gomori's reticulum stain, original magnification ×400).

forming a star-shaped lesion (Fig. 6). The cells forming the granuloma included clusters of large histiocyte-like cells and macrophages containing PAS positive material and hemosiderin. The histiocyte-like cells (Fig. 7) were large with irregular and indented nuclei with occasional prominent nucleoli. The cytoplasm appeared pale and acidophilic with ill-defined short dendrites extending from the cell membrane.

Some bronchioles adjacent to the inflammatory lesions were filled with acute inflammatory exudate (Fig. 5) and the alveolar spaces contained a marked number of alveolar macrophages (DIP-like reaction, Fig. 8). The alveolar epithelium was hyperplastic and the subpleural airspaces showed focal emphysematous changes.

By the end of 20 wks, 2 of 6 mice showed marked bronchial epithelial hyperplasia together with squamous metaplasia with occasional atypical epithelium. Multifocal interstitial granulomatous lesions were observed in the lungs of 2 of 6 mice similar to that found after 16 wks. Older interstitial lesions demonstrate typical satellite or star-shaped fibrosis.

Neoplastic-like lesions were found in the lungs of 2 mice. One lesion consisted of proliferating alveolar epithelial cells bounded by chronic inflammation (Fig. 9). These histopathologic features are similar to so called alveogenic lung tumors, although the nature of these lesions is in question, ie reactive or neoplastic.

The lesion in the lung of the second mouse was a single microscopic lesion consisting of multiple papillary structures (Fig. 10), filling what appeared to be a bronchiolar lumen. The cells of this lesion were cuboidal to columnar in shape and the nuclei hyperchromatic, rounded and eccentrically located. There were no cilia extending from the free borders of these cells. The histologic features of this lesion were consistent with a Clara cell bronchial adenoma.

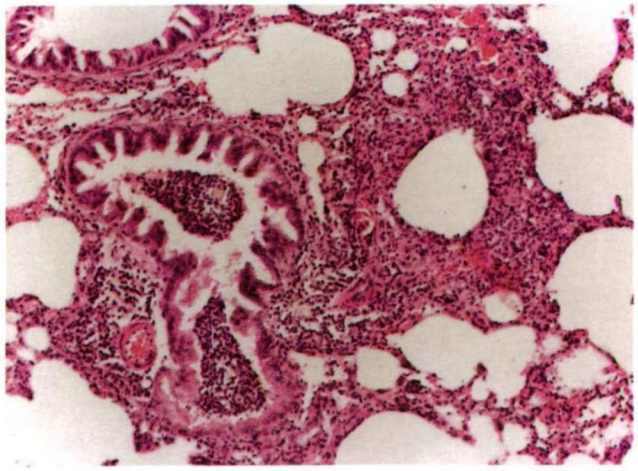

Fig. 5 Interstitial pulmonary granuloma with a central cavity. Polymorphs are present in the bronchial lumen. (Haematoxylin and eosin, original magnification ×200).

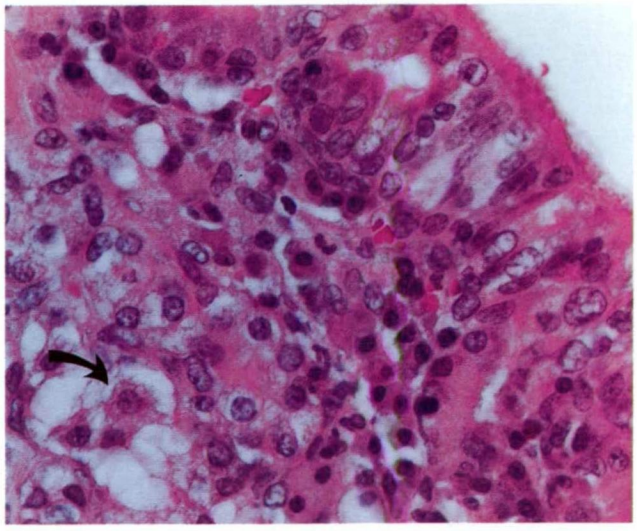

Fig. 7 Langerhans dendritic cells (arrow) in peribronchial interstitial granuloma. (Haematoxylin and eosin, original magnification ×400).

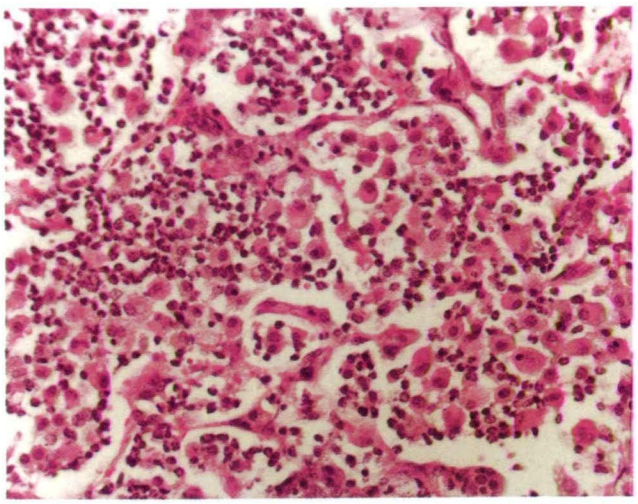

Fig. 8 Desquamative interstitial pneumonia (DIP) in the lung parenchyma. (Haematoxylin and eosin, original magnification ×100).

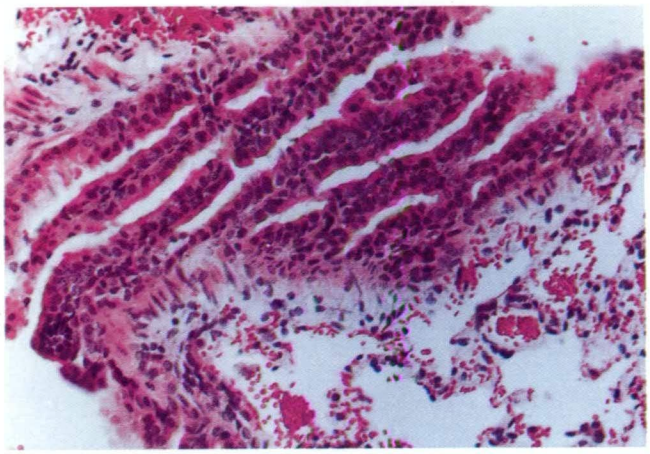

Fig. 10 Papillary Clara cell adenoma. (Haematoxylin and eosin, original magnification ×400).

At week 20, tobacco smoke exposure ceased and the remaining mice (6) were kept for a further 6 wks. The alveolar epithelial hyperplasia, the interstitial cellular accumulation, interstitial granulomas, features of bronchiolitis, DIP and the emphysematous changes resolved in 83% of the mice (5/6). A mild degree of these changes were observed in the remaining mouse. Only bronchial epithelial metaplasia persisted.

Control animals did not show any of the changes observed in the test group during the time course of the study.

Pulmonary ZIO positive Langerhans cells

The ZIO positive dendritic cells were smaller than alveolar macrophages and most were oval or triangular in shape. Black granules (Fig. 11) were observed scattered in the cytoplasm. The dendritic nature of these cells was demonstrated in serial sections. The nuclear:cytoplasmic ratio was always low around 1:2 and the nucleus appeared irregular. Most of the ZIO positive LC were found in the alveolar septum, and infrequent in alveolar spaces and were rarely found in the bronchial epithelium. The mean number of ZIO positive LC in normal lung was 1.3 LC ± 0.4/HPF.

There was a steady increase in the number of ZIO positive LC mainly in the thickened alveolar septa of the tobacco smoke exposed mice (Fig. 12) starting from the end of the first week (test = 2.3 ± 1; control = 0.8 ± 0.3). After 8 wks of tobacco smoke exposure, the number of pulmonary LC peaked (test = 16.5 ± 3.6; control = 0.67 ± 0.4, $p < 0.0001$) and remained high with continuous exposure to tobacco smoke (test = 14.3 ± 4.3; control = 0.5 ± 0.2, $p < 0.0001$). When exposure to tobacco smoke ceased at the end of the 20 wks, the number of ZIO positive LC in the lungs of the remaining mice decreased to the level of the control group (test = 0.5 ± 0.3; control = 0.7 ± 0.6) after a further 6 wks.

On ultrastructure the pulmonary dendritic cells with positive ZIO staining showed all the ultrastructural features of LC; the indented large irregular nucleus and

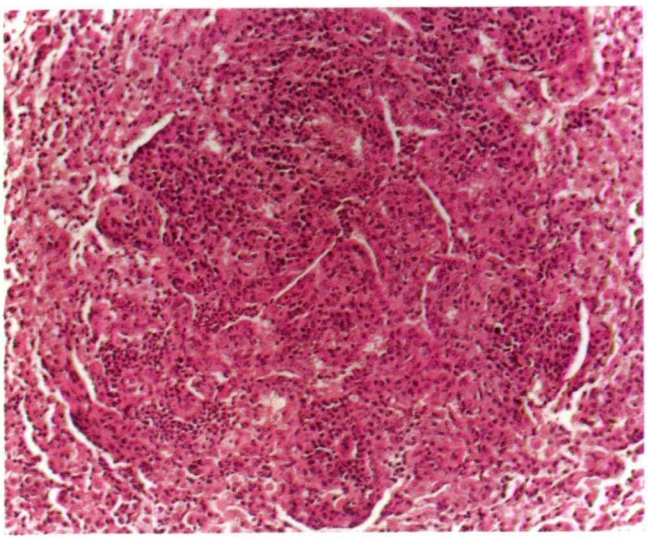

Fig. 9 Focal alveolar proliferative lesion bounded by inflammatory reaction. (Haematoxylin and eosin, original magnification ×200).

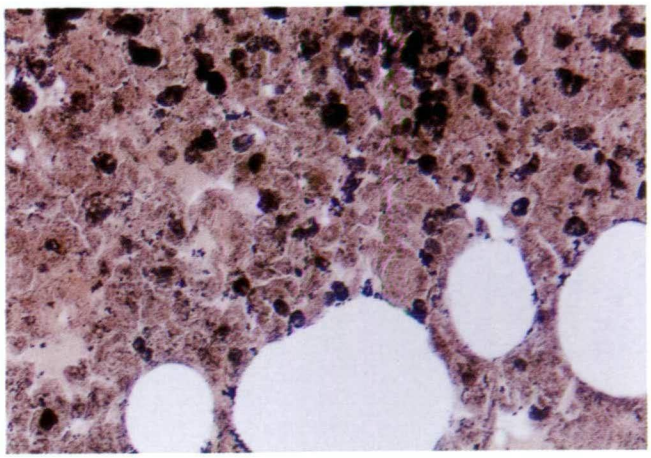

Fig. 11 ZIO positive dendritic cells in lung granulomatous inflammation after exposure to tobacco smoke. (ZIO staining, original magnification ×200).

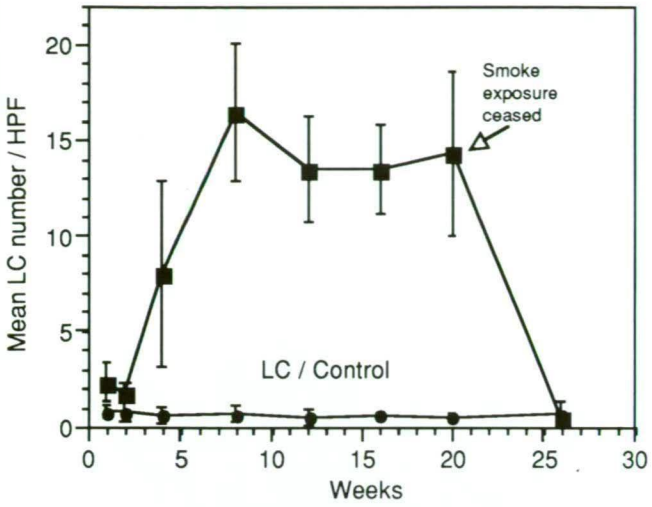

Fig. 12 Pulmonary murine LC after exposure to tobacco smoke. Analysis of variance between test and control group showed a statistically significant difference in LC number ($p < 0.0001$) between 4-20 wks.

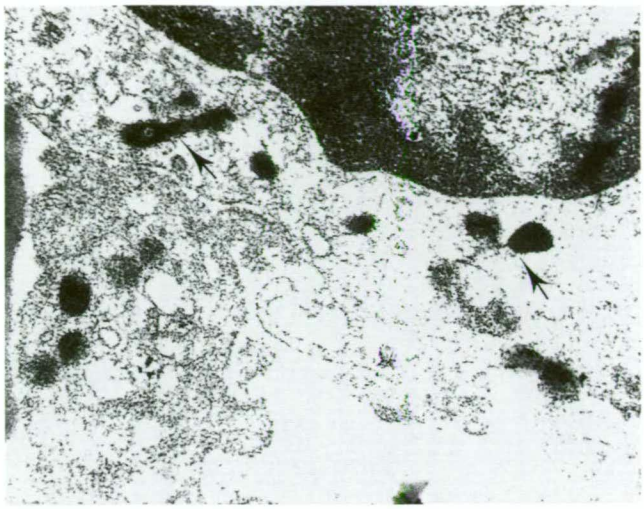

Fig. 14 ZIO positive pulmonary dendritic cell showing branched and unbranched labelled (arrow) Birbeck granules. (V-7.4-ZIO staining, original magnification ×34,000).

black BG were easily identifiable (Figs. 13 and 14). Some branched BG were observed near the nuclear membrane. Many tubular structures were identified in the cytoplasm and the mitochondria were numerous.

Ia positive pulmonary LC

The morphology and size of the Ia positive dendritic cells were consistent with that of ZIO positive LC. Ia positive cells were mainly found in the alveolar septum and were occasionally seen with a few dendrites. Their numbers were similar to that of the ZIO positive LC.

DISCUSSION

The number of dendritic LC in the lungs of mice exposed to tobacco smoke for 20 wks increased approximately 25 fold. Although the increase in the pulmonary LC was observed within one wk, the highest level was obtained after 8 wks and remained elevated until smoke inhalation treatment ceased at 20 wks. This is in agreement with Casolaro et al.,[8] and Soler,[9] who reported an increase in LC in smokers' lungs in humans.

In this study ZIO positive pulmonary LC were always observed in control lungs in the alveolar septum and rarely in the bronchial epithelium. The mean of ZIO positive pulmonary LC in normal murine lung was 0.9 ± 0.4 cells/HPF. This parallels our previous findings on the low density of pulmonary LC in normal human lung.[26]

The increase in the ZIO positive pulmonary LC after tobacco smoke exposure was associated with the development of interstitial granulomas and inflammation. The alveolar spaces around the granulomas showed alveolar epithelial hyperplasia with marked accumulation of alveolar macrophages. By 16 wks, the basic lesion was the interstitial granuloma with a central cavity together with an inflammatory reaction of the lung parenchyma around the granuloma. The cellular infiltrate of the granuloma extended into the adjacent alveolar septum forming star-shaped lesions.

The cytological features of the histiocyte-like cell were consistent with those of the histiocytosis X cell.[11] They were irregular, with an indented or lobulated nucleus, pale acidophilic cytoplasm with ill-defined short dendrites extending from the cell membrane. While there were many of these cells in active lesions, they become rare in older fibrotic lesions.

In this study, on ultrastructural examination of the ZIO positive pulmonary dendritic cells, BG (Figs. 13 and 14) were observed in the cytoplasm. This confirmed the lineage relationship between histiocytosis X cell and LC.

The bronchioles associated with the granulomas were filled with acute inflammatory exudate and the alveolar spaces contained many alveolar macrophages (DIP-like reaction). The alveolar epithelium was hyperplastic. By the end of 20 wks, some older interstitial granulomas progressed into typical fibrotic satellite or star-shaped lesions. These histopathological features are consistent with the human clinical syndrome related to tobacco

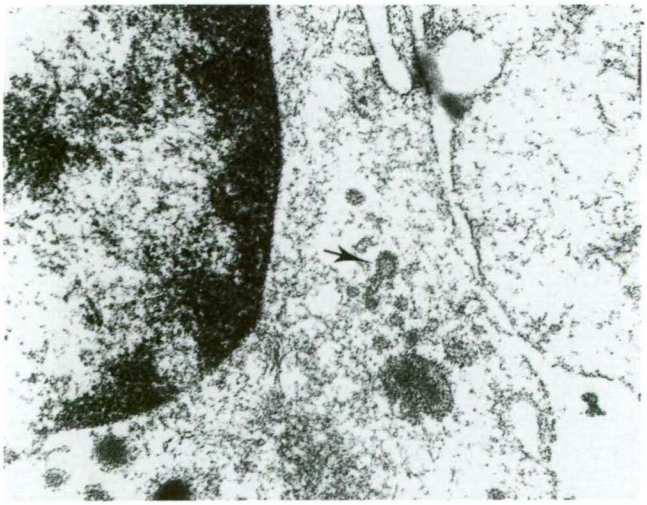

Fig. 13 ZIO positive pulmonary dendritic cell showing labelled (arrow) Birbeck granules. (V-7.4-ZIO staining, original magnification ×34,000).

smoke, pulmonary histiocytosis X,[10,11] now called pulmonary LC granulomatosis.[12]

Although the pathogenesis of pulmonary LC granulomatosis is not clear, 90% of patients are smokers.[10-13] Carcinogens of tobacco smoke, eg benzo[a]pyrene and catechols may cause alveolar epithelial damage leading to proliferative, hyperplastic and neoplastic alveolar epithelial cells.[10,20,27,28] The damaged alveolar epithelium may express neoantigens which trigger the LC response and T-lymphocyte activation leading to necrosis of the target cells. Activated macrophages secreting collagenase, elastase, prostaglandins and other factors are also central to granuloma formation.[10,20]

Benzo[a]pyrene (BP) while increasing the number of pulmonary LC, may alter cellular immunity via prostaglandins.[29,30] Ruby et al.,[29] and Andrews et al.,[30] reported BP and co-carcinogen catechols increased the number of Ia positive LC, changed their morphology and impaired their function. This was associated with the development of skin tumors after 24 wks of treatment. Andrews and colleagues,[30] implanted the prostaglandin synthetase inhibitor, indomethacin beneath mouse skin before treatment with BP. This restored contact hypersensitivity; delayed the onset and reduced the size of skin tumors induced by BP. In a separate study we also investigated tobacco smoke condensate (TSC) induced cutaneous carcinogenesis and changes in epidermal LC. TSC increased the LC density, altered their morphology and impaired their function. This was associated with skin tumor development.[28]

Studies on the carcinogenic effects of tobacco smoke on the lungs of laboratory animals have concentrated on precancerous changes and tumor development.[3,4,31] Interstitial granulomas have been observed only in one previous study, ie that by Mostofi and Larsen.[31] They were investigating the histopathogenesis of urethane induced pulmonary tumors in mice when they observed the formation of interstitial granulomas between 3-12 wks prior to tumor development. They also observed large acidophilic cells in these granulomas. While Mostofi and Larsen,[31] did not investigate their observation further, their description of the interstitial granuloma is consistent with that of pulmonary LC granulomatosis (histiocytosis X). The large acidophilic cells are atypical LC.[32]

In this study we observed that the development of tumor-like lesions followed the formation of the interstitial granuloma after 20 wks exposure to tobacco smoke. The histopathologic features of these lesions were consistent with bronchial Clara cell adenoma and so called alveogenic tumors. This was associated with a high density of ZIO positive pulmonary LC. Shimkin and Stoner,[32] reported 95% of lung tumors in mice originate from the alveolar epithelium. We have previously described high density of S100 positive LC in tobacco smoke related human bronchioalveolar carcinoma and well differentiated squamous cell carcinoma.[26]

While tobacco smoke increased the ZIO positive pulmonary LC density, the impaired function of these cells induced by benzo[a]pyrene in the inhaled tobacco smoke may have resulted in disordered alveolar epithelial growth in a setting of compromized immunological function.

After tobacco smoke exposure ceased the histopathological changes resolved and the density of pulmonary LC returned to that of control levels. Only the bronchial epithelial metaplasia did not show reversible change. Bronchial epithelial metaplasia may require a longer time to resolve.

In conclusion, this study has demonstrated that tobacco smoke increased the density of ZIO positive pulmonary dendritic cells and this was associated with the development of bronchial squamous metaplasia, interstitial granulomatous lesions (pulmonary LC granulomatosis) and alveolar cell proliferation. The density of pulmonary LC returned to that of the control level after ceasing tobacco smoke exposure and the interstitial granulomatous lesions, but not the metaplasia resolved. The mechanism by which tobacco smoke increased the density of pulmonary LC may be related to benzo[a]pyrene, while functional changes in these antigen presenting cells may have a role both in chronic granulomatous inflammation and tumor development.

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Address for correspondence: H.K.M., Department of Pathology, University of Tasmania, 43 Collins Street, Hobart, Tasmania 7000, Australia.

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Tobacco smoke condensate cutaneous carcinogenesis: changes in Langerhans' cells and tumour regression

N.A. ZEID*† AND H.K. MULLER*

*Departments of *Pathology and †Surgery, University of Tasmania, Hobart, Australia*

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Summary. Tobacco smoke condensate was painted on the skin of BALB/c mice. It increased the density and changed the morphology of Langerhans' cells (LC). LC number in epidermal sheets of treated mice was significantly higher (1793 LC/mm^2) than in controls (946 LC/mm^2) ($P < 0.0001$) and remained elevated for 35 weeks. LC became less dendritic, or even rounded in shape, and smaller in size. The function of the morphologically altered LC was impaired when assessed by the contact hypersensitivity response. These changes were associated with skin tumour development in all treated mice.

Ten weeks after stopping the TSC treatment, LC number in skin tumours and in skin around these lesions had not decreased, but significantly increased ($P < 0.0001$). During this period tumour regression occurred in 23% of tumours; the remaining tumours showed a 50% reduction in size. At 45 weeks, the LC number in epidermal sheets around skin papillomas was 2274 ± 14.14/mm^2 and in invasive squamous cell carcinomas was 2088 ± 183/mm^2. This was associated with reversible changes in LC morphology, where cells became fully dendritic. This also correlated with lymphocytic infiltration into tumours, tumour necrosis, reduction in tumour size and/or tumour regression. It is concluded that the influx of normal LC into the skin tumours allowed the development of an immune response with tumour regression.

Keywords: Langerhans' cells, tobacco smoke condensate, carcinogens, squamous cell carcinoma, tumour regression

Benzo[a]pyrene and catechols are the most important of the 71 carcinogens in tobacco smoke condensate (TSC) (Dube & Green 1982). Skin papillomas and squamous cell carcinomas of the skin have been reported in various animals, e.g. guinea-pigs, Swiss mice, C57 black mice, after treatment with TSC (Wynder *et al.* 1953; Wynder 1961; Druckrey 1961; Day 1966).

Correspondence: Professor H.K. Muller, Department of Pathology, University of Tasmania, 43 Collins Street, Hobart, Australia 7000.

Ruby *et al.* (1989) and Andrews *et al.* (1991) studied the effect of the tobacco derived benzo[a]pyrene (BP) and co-carcinogen catechols on the number, morphology and function of epidermal Langerhans' cells (LC). They reported a significant increase in number of the Ia positive LC, changes in their morphology and impairment of their function. This was associated with the development of skin tumours after 24 weeks of treatment (58% were squamous cell papilloma and 42% squamous cell carcinoma).

The various reports on the density of LC in squamous cell carcinomas of human skin are conflicting. Gatter *et al.* (1984) used NA1/34 monoclonal antibody to identify LC in human skin tumours and reported changes in LC morphology and depletion in their number in squamous cell carcinomas. Smolle *et al.* (1986) used OKT-6 to study LC in human skin tumours and reported a low density of LC in squamous cell carcinomas compared to normal skin. McArdle and co-workers (1986) used an antibody to S100 protein to study LC in various skin lesions and reported a high LC density in keratoacanthoma, squamous cell carcinomas and basal cell carcinomas compared to the normal epidermis. Korenberg and his colleagues (1988) also used an antibody to S100 protein to study LC and supported McArdle *et al.* (1986) in regard to the high density of LC in keratoacanthoma but found a low density of S100 positive LC in squamous cell carcinomas. Alcalay *et al.* (1989) used adenosine triphosphate to study LC and reported changes in LC morphology but no increase in LC number in squamous cell carcinomas.

Analyses of LC in cervical intraepithelial neoplasia likewise are conflicting. The majority of reports indicate high LC number in cervical intraepithelial neoplasia (Morris *et al.* 1983, Caorsi & Figueroa 1984; 1986; Bonilla-Musoles *et al.* 1987; Xie X. 1990) but Tay *et al.* (1987) reported low LC numbers in this lesion. The variation in the number of LC in squamous cell carcinoma in these reports may reflect the different methods used including counting techniques.

The present investigation arose out of a previous study where we demonstrated an increase in LC density in well and moderately differentiated human lung squamous cell carcinomas in association with increased patient survival (Zeid & Muller 1993). As tobacco smoke is linked to the cause of these tumours (Auerbach *et al.* 1957), the present investigation was designed to examine the effect of TSC on LC during carcinogenesis in murine skin, a well established model to analyse the sequential development of squamous tumours.

Materials and methods

Animals

Two hundred and four BALB/c male mice 6–8 weeks old were obtained from the central animal house at the University of Tasmania after ethical approval. A group of mice (48) were used to assess the function of LC, while the remaining 156 mice were divided into two groups of 78 mice each, experimental and control. Control mice were treated with olive oil and acetone, while the experimental mice were treated with TSC in acetone and olive oil.

Experimental design

After shaving the dorsal skin of control mice, this group was treated with 200 μl of olive oil and acetone (1 : 1). The skin of the experimental mice was prepared as for the control and treated with 100 μl of olive oil and 100 μl of tobacco smoke condensate in acetone (1 : 1). The treatment was applied every second day including the weekends. At 35 weeks TSC treatment was stopped, and the mice were kept for a further 10 weeks to observe changes in tumour size and relation to LC numbers. Six mice from the control and test group were killed by cervical dislocation at 3 days, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 45 weeks. Skin sections were stained with haematoxylin and eosin and immunoperoxidase anti-Ia staining to visualize and count LC in the prepared epidermal sheets.

Preparation of tobacco smoke condensate

The TSC was prepared by condensation of cigarette smoke by a cold trap (Bentley & Burgan 1961). TSC was collected in jars and the water content evaporated by placing the jars, after gradual warming, in a water bath maintained at 100°C. TSC was then dissolved in an equal volume of acetone. Each 550 cigarettes gave 55 g (35 ml) of condensate. After dilution with an equal volume of acetone, the total amount of diluted condensate was 70 ml.

Preparation of epidermal sheets

A modification of the method of Scaletta and MacCallum (1972) was used to prepare epidermal sheets. The dorsal skin was shaved using electrical clippers and then depilated with a thioglycolate cream (Veet, Reckitt & Colman, UK). Adhesive cellophane tape was then applied to the dry skin, the skin was removed and incubated at 37°C for 3 hours in tetra-sodium ethylene-diamine tetraacetic acid solution (0.38 g EDTA in 50 ml PBS, pH 7.4; Serva, 11282). Using fine forceps the epidermis was separated from the dermis and stained with anti-Ia to demonstrate epidermal LC.

Immunoperoxidase anti-Ia staining of LC in epidermal sheets

Epidermal sheets were fixed in acetone at room

temperature for 20–30 minutes and then incubated overnight at 4°C with mouse monoclonal anti-Ia (MK-D6). After the sheets had been washed in PBS for 20–30 minutes, they were incubated at room temperature for 2 hours with horse-radish peroxidase-conjugated rabbit anti-mouse immunoglobulin (HRPO-anti-Ig, Dakopatts, Denmark), diluted 1:80 in PBS, pH 7.4. Epidermal sheets were washed again in PBS for 30 minutes and incubated for 10 minutes with diaminobenzidine (DAB; Sigma; 0.5 mg/ml of PBS containing 0.02% hydrogen peroxide, pH 7.4). The epidermal sheets were mounted in glycerine gel after washing in tap water.

The Ia positive LC were identified in the epidermal sheets as brown dendritic cells with various numbers of dendrites extending from the cell body (Ruby *et al.* 1989).

Immunoperoxidase anti-Ia staining of LC in tumour sections

Frozen sections (8 μm thick) of skin tumours were fixed in acetone for 20–30 minutes, washed three times in PBS over 20–30 minutes, incubated with anti-Ia for 30 minutes at room temperature, washed in PBS as before and incubated with HRPO-anti-Ig for 30 minutes at room temperature. Sections were washed again in PBS before incubation with DAB at room temperature (Ruby *et al.* 1989) for 10 minutes and counterstained in Mayer's haematoxylin after washing in running tap water for 5 minutes. Finally, sections were mounted in glycerine gel.

Enumeration of Ia positive LC in epidermal sheets

LC were assessed as the number of Ia positive LC/mm^2. The mean and the standard deviation were calculated.

Histology

Skin sections of mouse tumours and normal control skin were processed for routine haematoxylin and eosin staining.

Contact hypersensitivity assessment

The functions of LC were assessed by inducing a contact hypersensitivity reaction at the 2nd and the 6th weeks, after either treatment with TSC in a 1:1 solution of acetone:olive oil or, in the control mice, acetone:olive oil alone. The dorsal skin of the test mice was initially sensitized with 10 μl of a 1% solution of 2,4,6-trinitro-chlorobenzene (TNCB; Tokyo-Kasei Lot FCV61, 0.01 g/ml).

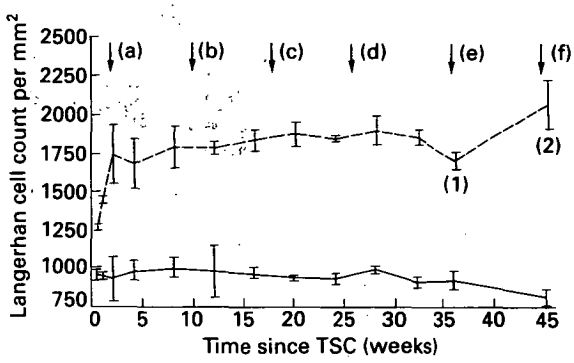

Figure 1. Skin lesions and changes in LC number induced by TSC. a, Epidermal hyperplasia; b, dysplastic changes; c, skin papilloma; d, early invasive squamous cell carcinoma; e, treatment with TSC stopped; f, 10 weeks post ceasing TSC treatment. Difference in LC number in epidermal sheets of both – – –, test and ——, control groups is significant ($P < 0.0001$). The increase in LC number in the test mice, 10 weeks after stopping TSC treatment (2) is significant compared with LC number at 35 weeks (1), $P < 0.0001$.

The right ears of both control and test mice were challenged with 10 μl of a 1% TNCB solution 5 days after sensitization. The thickness of the right and the left (unchallenged) ear was measured 24 and 48 hours later using a spring-loaded engineers' micrometer. The percentage increase in ear swelling was calculated according to the following formula:

$$\% \text{ increase} = \frac{\text{thickness of right ear} - \text{thickness of left ear}}{\text{thickness of left ear}} \times 100$$

The average percentage increase and the standard deviation were calculated for each group of mice.

Results

Skin pathology induced by tobacco smoke condensate (TSC)

The histopathological changes induced by the TSC in the skin of the treated mice are summarized in Figure 1. Epidermal hyperplasia was observed 2 weeks after treatment; the epidermis was composed of 6–8 cell layers compared to 2–3 layers of control epidermis.

Dysplastic changes in the hyperplastic epidermis were observed in 25% of mice after 10 weeks of TSC treatment. The cells had enlarged hyperchromatic nuclei, and a high nuclear:cytoplasmic ratio. By 18 weeks, about one-third of the treated mice developed macroscopic skin tumours, small wart-like papillomas, up to 2 mm in diameter.

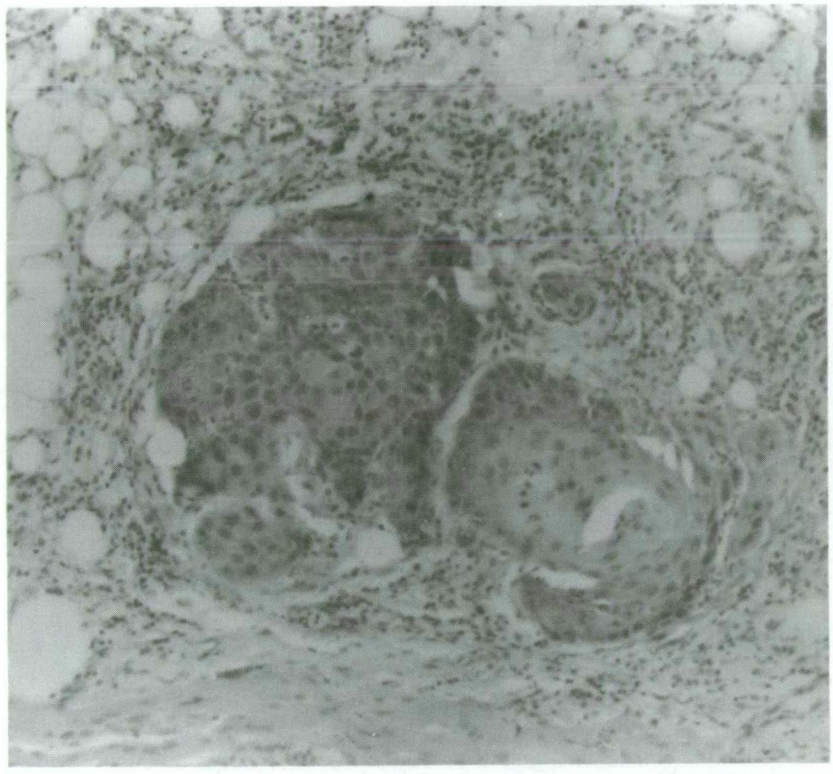

Figure 2. Nest of invasive squamous cell carcinoma with mononuclear cell reaction around tumour; 10 weeks after ceasing TSC treatment. H&E. ×200.

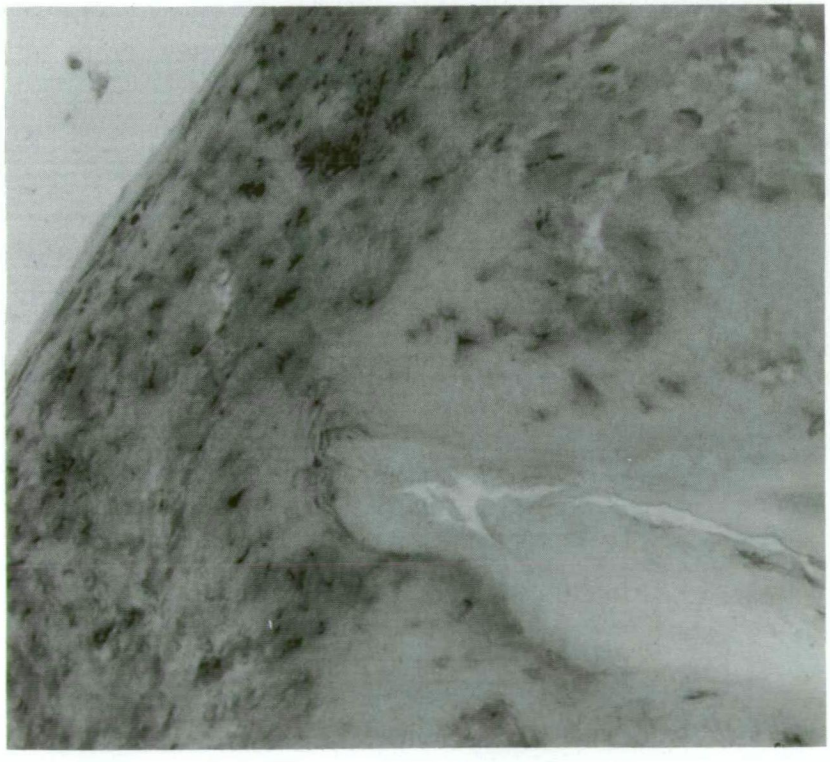

Figure 3. Ia positive LC in frozen section of skin tumour. Immunoperoxidase anti-Ia staining. ×200.

After 26 weeks of treatment, the skin of the remaining 50 mice showed skin tumours of various sizes, between 1 and 6 mm in diameter. The histology of the largest skin tumour showed early invasive squamous cell carcinoma. By 35 weeks, the sizes of the skin tumours of all treated mice were between 0.2 and 1.3 cm in diameter and one-third of the mice had developed multiple tumours. The histology of skin tumours of the six mice killed at this stage showed invasive squamous cell carcinoma.

Application of TSC was stopped at 35 weeks and 48 mice were kept under observation for a further 10 weeks. Macroscopically, 23% of tumours spontaneously disappeared and the remaining tumours showed about 50% reduction in their size. Histopathologic examination of these lesions revealed invasive squamous cell carcinomas of the skin in 50% of lesions with varying degrees of tumour necrosis and lymphocytic infiltration (Figure 2). Skin papillomas were observed in 27% of skin lesions; the remaining 23% showed various degrees of hyperplasia and dysplasia.

Epidermal LC enumeration in tobacco smoke condensate treated mice

During the first two weeks there was an increase in the density of the Ia positive LC in the skin of mice treated with TSC and the highest LC level was achieved 28 weeks after treatment. The number of LC in the skin of the treated mice during this period was significantly higher (1793 LC/mm^2) than in the controls (946 LC/mm^2) ($P < 0.0001$) (Figure 1).

At 45 weeks, almost 10 weeks after stopping the treatment with TSC, the number of LC in frozen skin tumours (Figure 3) and in epidermal sheets from skin around the lesions (Table 1) had not decreased and in general remained elevated (Figure 1). The exceptions were hyperplastic and dysplastic lesions. The difference in LC number before and after stopping TSC treatment was significant ($P < 0.0001$).

Table 1. LC in epidermal sheets around skin lesions 10 weeks after ceasing TSC treatment

Skin lesion	No.	Mean of LC/mm^2	s.d.
(1) Skin papilloma	13	2274	14.14
(2) Invasive SCC	13	2088	183
(3) SCC with necrosis	11	1960	61.25
(4) Hyperplasia and dysplasia	11	1650	153.53

Using Scheffe's test at the 0.05 level, 2 *vs* 1, 3 *vs* 1, 1 *vs* 2 and 1 *vs* 3 no significant difference; 4 *vs* 1, 2 and 3 all significant.

Table 2. Cutaneous contact hypersensitivity response to TNCP of mice treated with TSC

Group	No.	Time	Mean increase in ear thickness (%)
After two weeks			
Control	6	24 HR	110 ± 19.96
TSC	6	24 HR	54 ± 9.95
Control	6	48 HR	81 ± 6.96
TSC	6	48 HR	42 ± 8.06
After 6 weeks			
Control	6	24 HR	105 ± 6.59
TSC	6	24 HR	54 ± 9.95
Control	6	48 HR	76 ± 7.07
TSC	6	48 HR	50 ± 7

Differences between the percentage increase in ear thickness of test and control group is significant ($P \ll 0.0001$) at all times.

Epidermal LC morphology in tobacco smoke treated mice

After 2 weeks of TSC treatment, 25% of LC in epidermal sheets became smaller in size and the dendrites were blunt and short when compared with normal LC (Figure 4a). By 6 weeks many of the LC cells were rounded or oval in shape (Figure 4b).

Ten weeks following cessation of application of TSC the morphological changes reversed and the LC became more dendritic with more than 3 branched dendrites (Figure 5).

Contact hypersensitivity response after TSC treatment

Table 2 summarizes the percentage increase in ear thickness of both TSC treated and control mice at 2 and 6 weeks following sensitization and challenge with TNCB.

The percentage increase in thickness of the right ear of the TSC treated mice when challenged after sensitization with TNCB was significantly less than that of the control group ($P \ll 0.0001$), at both 24 and 48 hours. This suggests an impairment in the contact hypersensitivity response in the TSC treated mice and indicates a compromised function of the morphologically altered LC.

Discussion

TSC increased the density of the epidermal LC assessed in epidermal sheets; the highest LC level was achieved 28 weeks after treatment. The density of the epidermal LC in the treated skin was double the control value; the high level of the epidermal LC around lesions remained

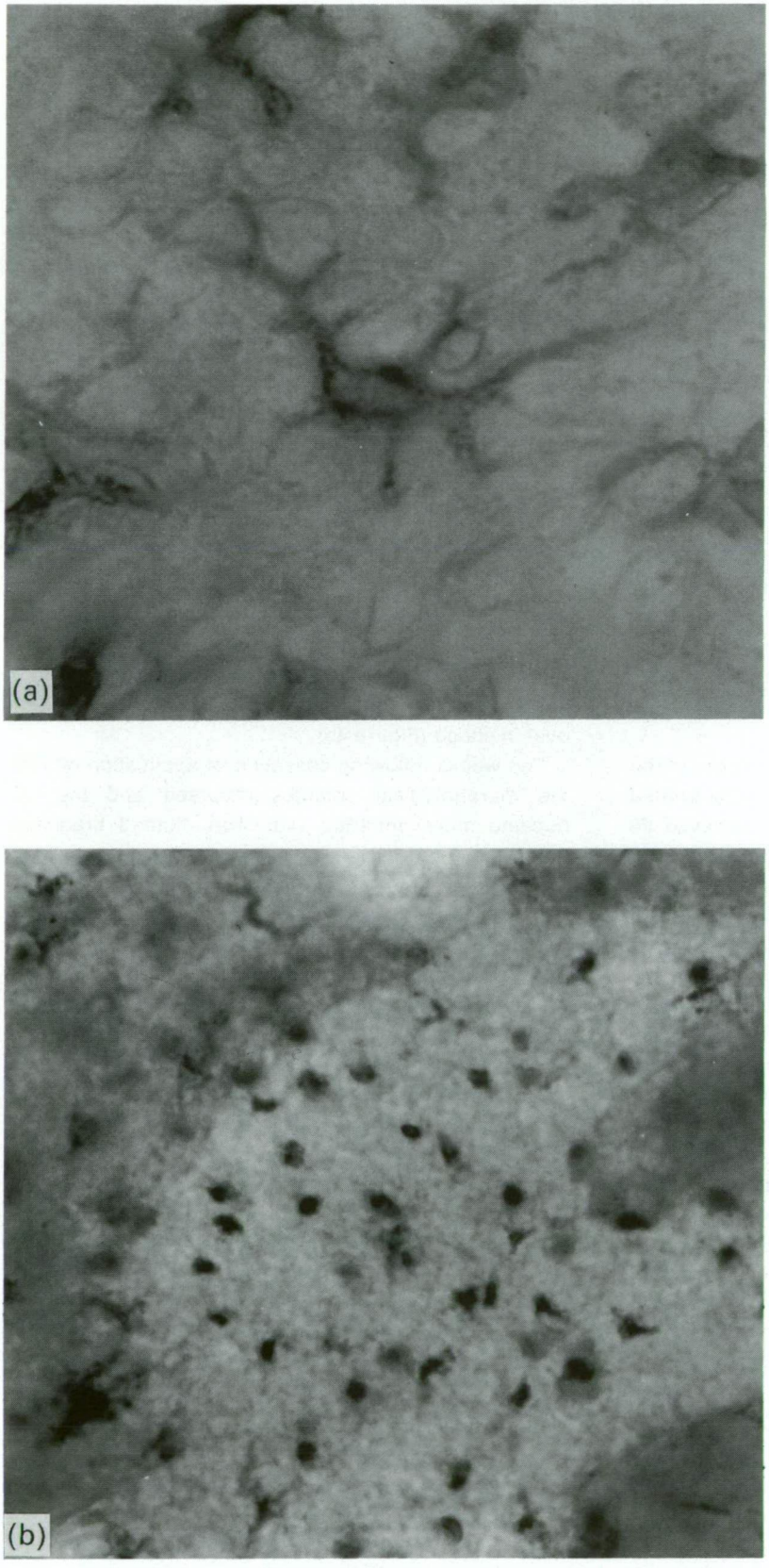

Figure 4. a, Normal fully dendritic LC in epidermal sheet of control mice. Immunoperoxidase anti-la staining. ×1000. b, Rounded LC 6 weeks after TSC treatment. Immunoperoxidase anti-la staining. ×400.

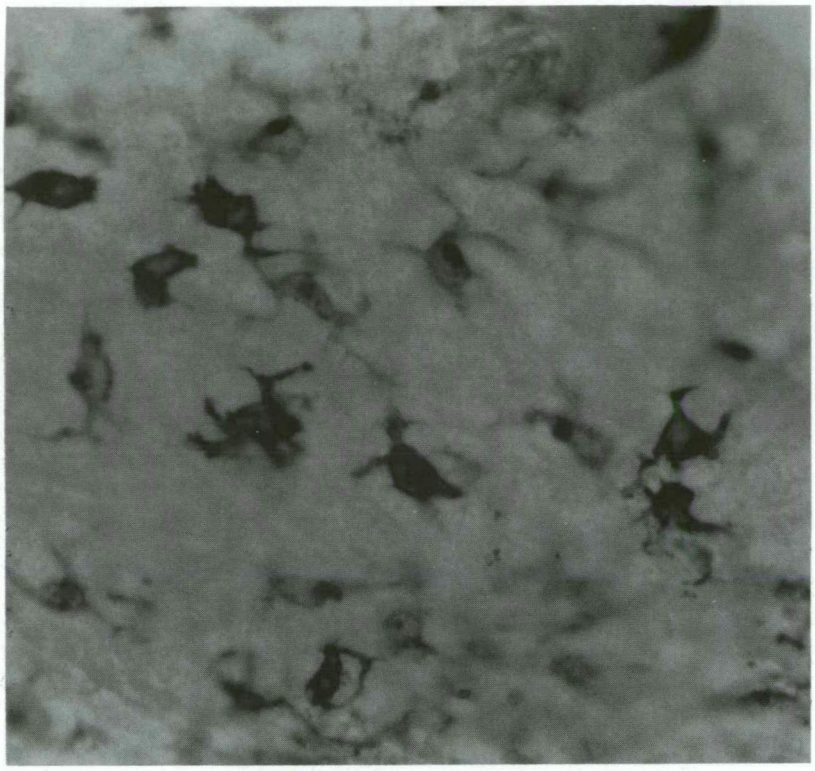

Figure 5. LC returned to their dendritic morphology, with more than 3 branched dendrites 10 weeks after stopping TSC treatment. Some LC still abnormal in shape. Immunoperoxidase anti-Ia staining. ×400.

elevated even after stopping TSC treatment. The morphology of epidermal LC in the treated skin was altered. Cells became smaller and the dendrites became shorter and blunt. The functions of the morphologically altered LC were impaired when assessed by the contact hypersensitivity response. The initial increase in epidermal LC number, the morphological changes and the impairment in their function observed during TSC treatment were also associated with tumour development.

These findings support similar observations reported by Ruby *et al.* (1989) and Andrews *et al.* (1991) when they investigated the effect of benzo[*a*]pyrene on murine epidermal LC. Andrews *et al.* (1991) demonstrated that implantation of the prostaglandin synthetase inhibitor, indomethacin, beneath mouse skin before treatment with the tobacco derived carcinogen, benzo[*a*]pyrene, delayed the onset of tumour development and reduced tumour size. Andrews and co-workers (1991) proposed that the cutaneous carcinogenesis induced by benzo[*a*]pyrene may be related to the suppression of cellular cutaneous immunity by prostaglandins.

It is of interest that while the density of epidermal LC decreased (1650 ± 154 cells/mm^2) in the skin of mice with hyperplastic and dysplastic lesions 10 weeks after stopping treatment with TSC, the density increased ($P < 0.0001$) in epidermal sheets around both squamous cell papillomas (2274 ± 14 cells/mm^2) and squamous cell carcinomas (2088 ± 183 cells/mm^2). The LC at this stage appeared fully dendritic. This was associated with spontaneous regression of 23% of skin tumours and 50% reduction in tumour size of the remaining lesions. The high density of the fully dendritic epidermal LC after stopping TSC application was accompanied by increased LC number in frozen sections of skin tumours. This was associated with marked lymphocytic infiltration and necrosis of these lesions.

Muller *et al.* (1985) also reported skin tumour regression after cessation of application of the chemical carcinogen, 7,12-dimethylbenz[*a*]anthracene (DMBA). DMBA depletes epidermal LC and this is associated with impaired skin immune function and skin tumour development (Muller *et al.* 1992). Repopulation of epidermal LC was associated with tumour regression (Muller *et al.* 1985).

The increase in LC density in epidermal sheets around tobacco smoke induced squamous cell skin carcinomas (Table 1) supports our previous report on the increase in S100 positive LC in squamous cell carcinomas of the lung (Zeid & Muller 1993). This is in agreement with the results of previous workers. McArdle *et al.* (1986) and Korenberg *et al.* (1988)

reported an increase in LC density in squamous cell carcinomas of the skin. Caorsi and Figueroa (1984), together with Bonilla-Musoles *et al.* (1987), reported a similar LC elevation in cervical squamous cell carcinoma, and Matsuda *et al.* (1990) in squamous cell carcinoma of the oesophagus.

LC are the best known antigen presenting cells. Their increase in certain tumours may follow recognition by the LC of antigen expressed by tumour cells. Grabbe *et al.* (1991; 1992) reported la positive LC are capable of presenting tumour associated antigen derived from a murine spindle cell tumour inducing an anti-tumour immune response manifested by tumour rejection and induction of delayed-type hypersensitivity in immunized animals.

While the extent of LC infiltration of cutaneous and other tumours may be linked to tumour antigens, how LC arrive in the skin lesions remains unclear. One possibility is that skin tumour derived cytokines attract LC into the skin lesions (Halliday *et al.* 1992). Once functionally normal LC are in lesions, they could trigger an immune response leading to tumour regression as in the present experiment.

It was concluded that TSC increased LC number, altered their morphology and impaired LC function; this was associated with tumour development. These events may be related to suppression of cellular cutaneous immunity by prostaglandins as related to the effects of benzo[a]pyrene already described. The further increase in epidermal LC number after stopping TSC treatment was associated with reversible changes in LC morphology; the cells became fully dendritic. These changes correlated with lymphocytic infiltration, tumour necrosis, reduction in tumour size and/or tumour regression, presumably due to an effective anti-tumour response.

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